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THE CENTRAL NERVOUS SYSTEM AND REGENERATION IN CRUSTACEA

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(*Received* 15 *March* 1952)

INTRODUCTION

It has been shown (Needham, 1945, 1946, 1950) that the rate of regeneration of appendages in *Asellus aquaticus* (L.) is dependent upon innervation, as in amphibia (Schotté & Butler, 1941, 1944; Singer, 1943) and in other groups of animals (Korschelt, 1927). The object of the present study was to obtain further information on the origin and nature of this neural factor, and in particular to ascertain whether it emanates from the segmental ganglion or from higher centres of the c.n.s. A trophic effect of the vertebrate nervous system on non-regenerating structures (Wyburn-Mason, 1950), which appears to be related to the effect of the system on wound-healing and regeneration, originates locally, since atrophy of organs in the vertebrates does not immediately follow spinal transection, but is correlated with the subsequent atrophy of the local nerve-centres (Wyburn-Mason, 1950, p. 192). There is evidence (Singer, 1943) that in amphibia the regeneration-effect likewise is thus localized.

To test this it is necessary to isolate the local centre from the rest of the c.n.s. The crustacea are ideal for this purpose since their cytons are virtually restricted to the segmental ganglia, and individual body-segments are easily isolated by transection of the intersegmental connectives. In the present experiments the regeneration of the right seventh thoracic limb (th. 7) in *A. aquaticus*, following degrees of such isolation of its segmental ganglion, has been compared with normal regeneration and again with regeneration following partial ablation of the segmental ganglion, without transection of the connectives. As an additional control, the rate of regeneration of the right sixth and eighth limbs was measured simultaneously. In this animal the exoskeleton is sufficiently transparent to permit operation through it.

METHODS

The general technique of operation and measurement has been described (Needham, 1945, 1949*b*). In the control series, of animals regenerating normally, the right limbs of thoracic segments 6, 7, 8 (subsequently designated th. 6, 7, 8) were removed at the autotomy-plane and allowed to regenerate under standard laboratory conditions. In a first experimental series the intersegmental nerve-connectives between th. 6 and 7 were severed between the edges of two fine steel needles, the ends of

which had been bent at right angles, tempered and sharpened. The tips were passed transversely to the nerve-cords from either side, one above, the other through the exoskeleton and below the cords. The lower needle was slightly curved at the tip to ensure retention of the cords during the lateral shearing action. On severance of the cords their cut ends sprang apart, and complete sensory and motor independence resulted between limbs anterior and posterior to the lesion. The right th. 6, 7, 8 were removed immediately prior to the operation. Mortality was high in this series and therefore a further series was subjected to incomplete transection, by merely crushing the connectives between the needle-blades. By this method varying degrees of functional isolation were obtained between the limbs on either side of the lesion, and persisted with little change during the period of regeneration studied. In these two series it was anticipated that the relative effects of transection on the regeneration of th. 6 and 7 would measure the relative importance for regeneration of any influences passing respectively forwards and backwards in the c.n.s. The effect on th. 8, relative to that on th. 7, was expected to show how far such influences are restricted to the immediate vicinity of the lesion. This was further tested by comparison with a fourth series in which the intersegmental cords were crushed between th. 7 and 8, as well as between th. 6 and 7. The main object of this last series, however, was the complete neural isolation of th. 7. Comparison with the preceding series also permitted an estimate of the general effect of increasing severity of operation (p. 155).

Partial ablation of the right segmental ganglion of th. 7, with the tip of a needle, is a simple operation. Under bright illumination the ganglion is easily visible, and it is held stationary between the ventral nerve cord and the peripheral nerve. The amount of damage to other tissues, and of haemorrhage, in this series was probably less than in the preceding experimental series. The right th. 6, 7, 8 were removed as before. In a further small group of individuals regeneration-rate was measured, for th. 6 and 7 only, in two successive acts of regeneration, following damage to the right 7th segmental ganglion.

The six series will be numbered and considered in the following order:

1. Normally regenerating (controls).
2. Intersegmental connectives crushed, th. 6 and 7.
3. Intersegmental connectives crushed, th. 6 and 7, th. 7 and 8.
4. Intersegmental connectives completely severed, th. 6 and 7.
5. Right segmental ganglion partially destroyed, th. 7.
6. Two successive regenerations following damage to right th. 7 ganglion.

The regenerating limbs were measured at eclosion and the mean eclosion-length (e.l.) computed for each of the three limbs, in each series. From this the specific daily mean increment in each (s.d.m.i.) was computed (Needham, 1949*a*) by dividing the mean e.l. by the product of the mean body-size and mean eclosion-time (i.e. the mean interval between operation and eclosion). This measure of specific regeneration-rate which involves relatively little labour was considered adequate for estimating general effects of the experimental treatments, and is justified by the precision with which it reveals general trends (Fig. 1).

Differential effects on the three limbs were estimated by a more precise method, the comparison of the observed values of e.l. 7 and e.l. 8 with those to be expected, in normal regeneration, from the observed values of e.l. 6. This comparison was made for each individual animal in each series, and the mean difference, expected/

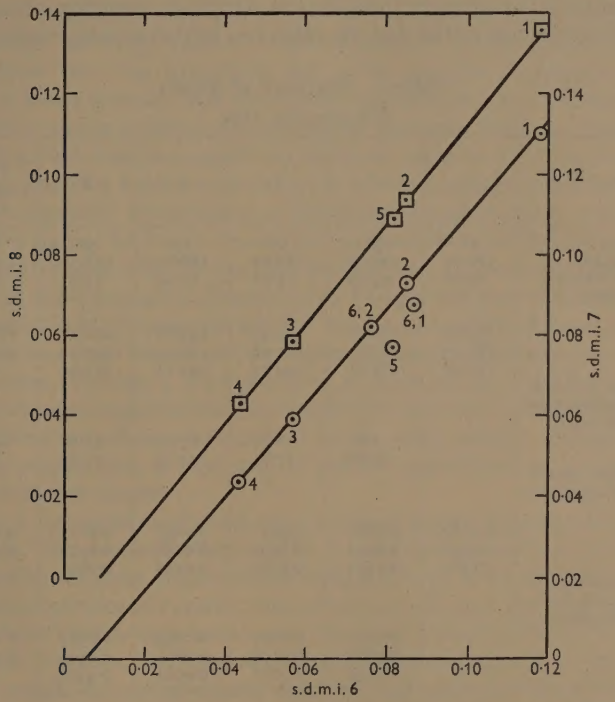

Fig. 1. Graph of specific regeneration-rates (s.d.m.i.) of th. 7 and 8 against that of th. 6, in the six series. The number of the series is entered by each point.

observed, for limbs 7, 8 was the property tested for significance. Variables common to the three limbs are eliminated by this method, which measures purely differential effects.

All measurements are quoted in the units mm. × 60. Abdomen-width was used as the measure of body-size.

RESULTS

The regressions of e.l. 6 and e.l. 8* on e.l. 7 in normal regeneration were:

$$\text{e.l. } 6 = (164{\cdot}52 \pm 1{\cdot}159) + (0{\cdot}898 \pm 0{\cdot}029)\ (\text{e.l. } 7 - 181{\cdot}202), \quad (1)$$

$$\text{e.l. } 8 = (190{\cdot}04 \pm 0{\cdot}504) + (1{\cdot}015 \pm 0{\cdot}012)\ (\text{e.l. } 7 - 181{\cdot}202), \quad (2)$$

* Initially it was considered that these regressions would be the most useful.

from which it may be calculated that:

$$\text{e.l. } 7 = 1{\cdot}113\ (\text{e.l. } 6) - 1{\cdot}567, \tag{3}$$

$$\text{e.l. } 8 = 1{\cdot}130\ (\text{e.l. } 6) + 4{\cdot}153. \tag{4}$$

It is clear that the independent term of equation 3 is not significantly different from zero, whereas that of equation 4 may be just significant, implying either that th. 8 begins its regeneration earlier than the other two or that its rate, relative to theirs,

Table 1. *Summary of Results*

(All units mm. × 60)

Series Number (see p. 152) ...	1	2	3	4	5	6 First regenera-tion	6 Second regenera-tion
Number of records	41	36	17	13	39	8	8
Mean abdomen-width	127·94	149·08	187·47	188·69	176·90	137·50	146·75
Mean eclosion-time (days)	10·90	13·50	13·61	16·46	13·95	13·50	14·88
Mean eclosion-length (e.l.) of:							
th. 6	164·52	170·56	145·57	135·86	201·17	160·88	166·79
th. 7	181·20	187·71	151·22	136·12	191·13	162·78	178·95
th. 8	190·04	188·31	149·25	133·15	219·94	—	—
Mean deficit of e.l. 7, e.l. 8, below values expected from observed value of e.l. 6:							
e.l. 7	—	1·29	8·89	13·55	31·25	14·58	9·83
e.l. 8	—	12·08	17·51	24·53	10·77	—	—
Specific daily mean increment (s.d.m.i.) in regeneration of:							
th. 6	0·1180	0·0847	0·0571	0·0437	0·0815	0·0867	0·0764
th. 7	0·1299	0·0932	0·0593	0·0438	0·0774	0·0877	0·0820
th. 8	0·1360	0·0936	0·0585	0·0429	0·0891	—	—
Difference between observed value of s.d.m.i. and that for normal regeneration:							
th. 6	—	0·0333	0·0609	0·0743	0·0365	0·0313	0·0416
th. 7	—	0·0367	0·0706	0·0861	0·0525	0·0422	0·0479
th. 8	—	0·0424	0·0775	0·0941	0·0469	—	—

is higher at the outset than later. The slope of the regression of e.l. 8 on e.l. 7 is not significantly different from 1·0, whereas that of e.l. 6 on e.l. 7 is significantly less than 1·0; after the initial irregularity, therefore, the rate of regeneration of th. 8 is strictly proportional to that of th. 7, whereas th. 6 regenerates more slowly, although it begins at the same time as th. 7. As a consequence the eclosion-lengths of the three limbs (Table 1) come to bear approximately the same proportions to their definitive lengths, which likewise show a smaller difference between 8 and 7 than between 7 and 6. The regressions of the definitive lengths of th. 6 and 8 on th. 7 are:

$$\text{th. } 6 = (246{\cdot}938 \pm 1{\cdot}014) + (0{\cdot}849 \pm 0{\cdot}010)\ (\text{th. } 7 - 290{\cdot}144), \tag{5}$$

$$\text{th. } 8 = (308{\cdot}521 \pm 1{\cdot}181) + (1{\cdot}077 \pm 0{\cdot}012)\ (\text{th. } 7 - 290{\cdot}144); \tag{6}$$

giving

$$\text{th. } 7 = 1{\cdot}180\ (\text{th. } 6) - 0{\cdot}873, \tag{7}$$

$$\text{th. } 8 = 1{\cdot}271\ (\text{th. } 6) - 4{\cdot}699. \tag{8}$$

That of th. 7 on th. 6 is not significantly different from that in normal regeneration,

and the difference in th. 8/th. 6 is to be expected from the fact that th. 8 is inhibited during early ontogenesis (Needham, 1937, p. 295). It is noteworthy that the regression of th. 8 on th. 7, in normal growth, nevertheless passes through the origin, so that the rapid growth of th. 8 in the post-inhibitory period (Needham, *loc. cit.*) very soon must bring its proportionate length to the definitive value, which is subsequently maintained throughout growth. The regression of th. 6 on th. 7 also passes through the origin, and a simple proportion is maintained throughout growth. Apart from the irregularity due to the peculiar ontogenesis of th. 8, therefore, it seems probable that the relative regeneration-rates of limbs are very similar to their relative growth-rates in ontogeny. However, while regeneration-rate, like normal growth-rate, is progressively less in the order th. $8 > 7 > 6$, the gradient is less steep, implying a relative advantage to the more anterior limbs, in regeneration. This is probably an indication of the differential effect which more strongly characterizes general inhibitory conditions in regeneration (see below).

In all experimental series (p. 152) the specific mean regeneration-rate (s.d.m.i., Table 1) was below normal in all three limbs. This general retardation has not been tested statistically in all series, since it was not the main object of the experiments, but it was an invariable feature, and its magnitude was correlated with the magnitude of the operation. In series 3, 4 it was so great as to obviate any need for quantitative tests. The absolute magnitude of this retardation increased in the order $6 < 7 < 8$ but the relative magnitude also increased in that order. Such a differential effect may be an exaggeration of that seen in normal regeneration as compared with normal growth (see above).

In series 2 (connectives crushed, 6–7 intersegment) the general inhibition was considerable. The relative inhibition of th. 7 was a little greater than that of th. 6, as measured by the mean (expected-observed) difference in e.l. (Table 1), and that of th. 8 was proportionately greater when allowance was made for two exceptionally inhibited limbs (which were included in the computation for Table 1). It seems reasonable to conclude, therefore, that transection of the connectives between two segmental ganglia did not specifically retard the regeneration of the limb on either side of the lesion (th. 6 and 7); there was merely a general inhibitory effect, acting differentially on the three limbs in the order of their normal regeneration-rates.

In series 3 (connectives crushed 6–7 and 7–8 intersegments) the general retardation of regeneration was nearly twice as great as in series 2 and was therefore proportional to the extent of the injury at operation. Further the gradient in relative inhibition along the series of limbs was correspondingly steeper, so that the absolute rate of regeneration of th. 8 was actually lower than that of th. 7 (Table 1). The values of e.l. 8 fell short of those to be expected from the observed values of e.l. 6 by a mean figure of $17{\cdot}5 \pm 5{\cdot}82$ units, which may be regarded as significant.

In series 4 (complete transection of connectives, 6–7 intersegment) the general retardation was even greater and the gradient in differential inhibition proportionately steeper, so that the absolute regeneration-rate of th. 8 was lower than that of both th. 7 and th. 6. E.l. 8 fell short of the value to be expected from the observed value of e.l. 6 by an average of $24{\cdot}53 \pm 7{\cdot}48$ units, which again is significant.

Although the amount of tissue destroyed in the operation for this series was less than in that of the previous series, haemorrhage was greater, and the interruption of the c.n.s. was complete and permanent.

Comparing the results of the three transection-series it seems that there is no good evidence of a specific effect on the regeneration of any of the three limbs but only of a general inhibitory effect on all, which therefore was probably systemically mediated. It was a function of the magnitude of operational injury and a differential function of the normal regeneration-rate of the limb.

The results of series 5, 6 (partial ablation of 7th right segmental ganglion) contrasted sharply with those of the preceding series, showing, in addition to the general inhibition, a specific inhibition of th. 7. The general inhibition, as measured by the reduction in mean specific regeneration-rate of th. 6 and 8, was not significantly greater than in series 2, and the relative inhibition of th. 8, also, was virtually the same as in that series. By contrast the deficit of e.l. 7 below expectation, virtually zero in series 2, was highly significant in series 5 ($31{\cdot}25 \pm 4{\cdot}16$). The magnitude of the specific inhibition was greater, and its standard error less, than the greatest inhibition produced by complete transection. The effect was virtually restricted (Fig. 1) to th. 7, the specific regeneration-rate of which was absolutely less than that of th. 6 (Table 1).

For the eight individuals of series 6 the mean specific regeneration-rate of th. 6, in the first act of regeneration, was virtually the same as in the larger series (5). That of th. 7 was considerably reduced by the operation, though less than in the larger series, largely owing to variation in the degree of injury to the ganglion. In the second act of regeneration the mean specific regeneration-rate of th. 6 was lower than in the first act, no doubt due to the normal effect of age and of repeated regeneration (Needham, 1949*b*), but the decline for th. 7 was relatively less, and an improvement in innervation is implied. This could be due entirely to regeneration of cut fibres and partially damaged cytons and does not necessarily imply replacement of destroyed cells. The recovery was such that the residual inhibition of the limb was virtually that characteristic of general inhibition (Fig. 1).

DISCUSSION

The main conclusions to be drawn from the results are that the nervous control of regeneration-rate in this crustacean emanates from the neurons of the local segmental ganglion. Section of the intersegmental connectives has virtually no specific effect on the regeneration of those limbs supplied from the ganglia immediately fore and aft of this lesion, so that the higher centres and other parts of the c.n.s. are probably not involved.

It is scarcely possible in this small crustacean to ascertain which functional component of the segmental nerve-supply is most active in the promotion of regeneration. This problem is incompletely solved for the vertebrates. Singer (1943) finds that the dorsal root-fibres are most important in the regeneration of amphibian limbs, as in neuro-trophic activity (Wyburn-Mason, 1950), though the motor supply also has some effect (Singer, 1945) as again in the neuro-trophic effect on normal

muscle. The view of Wyburn-Mason (1950, p. 59) that the trophic effect in vertebrates is due to unmyelinated autonomic efferent fibres running in both dorsal and ventral roots, would simplify the picture and might assimilate the earlier conclusion of Schotté (1926) that the sympathetic system promotes regeneration of amphibian limbs. However, it cannot be reconciled at present with the evidence that the trophic fibres of the dorsal root are antidromic afferent fibres, with their cell-bodies in the dorsal root-ganglion (Fulton, 1943, p. 27).

A possible, if minor, role in regeneration must be envisaged for the cutaneous nerve-net of crustacea (Nusbaum & Schreiber, 1897; Holmgren, 1898). At present there is no direct evidence of this but it is interesting that epidermal components of the insect limb may regenerate in the absence of the segmental ganglion (Suster, 1933), which is essential only for the mesodermal structures.

The differential operation of the general inhibition observed in these experiments recalls the differential inhibition observed in the regeneration and ontogenesis of various animals by Child (1941) and others. There, also, inhibition was proportional in magnitude to the normal activity. In *Asellus* the most probable basis would seem to be a differential distribution of the response of local tissues to the level of some essential, distributed by the blood. If damage to the c.n.s. plays any part, it is through systemic mechanisms, for example through a disturbance of cardio-vascular control. The results seem to rule out any direct control by the c.n.s. of this general component of the inhibition, and this is important since in other groups there is much evidence (Child, *loc. cit.*) that a graded organization of the nervous system may determine and control morphogenetic gradients. In the present instance the nervous system is not necessarily ruled out as the initial determinant, in ontogeny, of the morphogenetic gradient, but it is not continuously necessary for its subsequent expression.

When the values of s.d.m.i. for th. 7 and 8 are plotted (Fig. 1) against those for th. 6, in the six series, a straight line graph is obtained in each case. These lines do not pass through the origin but indicate that with increasing general inhibition, first th. 8 and then th. 7 would be completely suppressed. In other words the limbs require a progressively higher threshold of systemic factor, in the order $6 < 7 < 8$, for the initiation of regeneration. To increments above this threshold, however, the proportionate responses of the three limbs are in the same order as the thresholds, and the proportionate responses are constant for all increments above the threshold. It is not yet clear if this is true for differential inhibition in general. Child and his school, working mainly with soft-bodied animals, have been more restricted to qualitative and simple geometrical representation of the phenomenon. However, complete suppression of the higher levels of a gradient, under conditions which permit some growth of lower levels, is a familiar result (Child, *loc. cit.*) and implies some such differential threshold, though not necessarily the linear proportionality between the responses of different parts of the gradient to supraliminal conditions.

In passing it may be noted that the present gradient has its high point posteriorly (cf. Needham, 1943) in the body.

On the graph of s.d.m.i. 7/s.d.m.i. 6 the points for series 5 and series 6 (first act) fall below the line fitting all other points, confirming very strikingly the conclusion (p. 156) that the effect of partial ganglionectomy on th. 7 was the only specific effect produced in the experiments.

The extent of powers of regeneration in the c.n.s. of crustacea merits further investigation. After damage to the segmental ganglion there was some improvement of regenerative power (p. 156), and after crushing the intersegmental connectives there was a progressive, though often incomplete, restoration of sensory and motor co-ordination across the lesion. Both of these recoveries could have been due entirely to regeneration of cell-processes, and as yet there is no good histological evidence that cell-bodies are replaced, as they are in lower invertebrates and in the urodeles.

SUMMARY

1. The neural influence on the rate of regeneration of a limb in *Asellus* emanates from the segmental ganglion. It is independent of other centres of the c.n.s.

2. Operations on the animal cause a non-specific retardation of all limbs regenerating immediately afterwards. The immediate cause is probably haemorrhage, since the effect is both systemic and proportional to the severity of injury.

3. This general retardation acts differentially on a series of limbs, in proportion to their normal regeneration-rates. It has been possible to obtain a quantitative measure of this 'differential inhibition'.

I am indebted to Professor A. C. Hardy, F.R.S., for the facilities I have enjoyed while carrying out this work and to Dr A. C. Willis for references to the subcutaneous nervous system in Crustacea.

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POLARIZED LIGHT STUDIES OF THE CORNEA

I. THE ISOLATED CORNEA

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The importance of the cornea and the peculiar circumstances of its transparency have stimulated research for many years, but despite its apparent suitability there have not been any adequate quantitative investigations by means of polarized light. We shall show in this and the following paper that this technique, besides yielding results which throw light on the ultrastructure and fibre arrangement of the tissue (Naylor, 1952), also affords a means of assessing changes in the intra-ocular pressure of the intact eye by quantitative measurements of the photo-elastic properties of the cornea.

The application of quantitative polarization techniques to biological tissues presents many difficulties; nevertheless, measurements can be carried out with great accuracy if the tissues can be suitably examined in a polarizing microscope (Swann & Mitchison, 1950). In the present case the ordinary microscope was of limited value, since individual corneal fibres cannot easily be isolated and changes in their birefringence with changing intra-ocular pressure can only be measured in the intact eye. Modifications of the usual techniques of measuring retardation were therefore required, and these may be of some interest to those concerned with measurements in biological tissues elsewhere.

A study of the corneal interference figure, and measurements of the retardation of the cornea suspended in a pressure chamber under near physiological conditions have already been described (Stanworth & Naylor, 1950). This study—in particular the finding that the retardation was essentially zero for light incident normally to the surface—led us to postulate that the cornea behaves as a curved uniaxial crystal plate with its optic axis perpendicular to its surface at all points, and that this could be explained qualitatively on the basis of a random orientation of the corneal lamellae. It is possible to show this quantitatively, and to deduce from the measurements the value of the birefringence of the individual corneal fibres.

From the present point of view the cornea consists of about 100 curved layers, each containing roughly parallel fibres. Each fibre has its optic (slow) axis along its length (His, 1856), and each layer, therefore, behaves as a crystal plate with its optic axis in the plane of the plate.

The calculation of the effect of such superimposed crystal plates on transmitted polarized light is extremely laborious; general formulae have been given by Mallard (1884) and Tuckerman (1909), but these are not easily applicable to the present case. In general, the effect of a series of plates depends not only on the character-

istics of the plates themselves, but also on their order, so that in the general case no single solution is possible. Mallard, however, has shown that if the plates are very thin, so that second and higher powers of thickness can be neglected, and if the total effect on the transmitted light is small, then the formulae can be considerably simplified. He has shown that such a series of plates acts for light passing in any direction as would a single plate of the same total thickness but having such characteristics that the emergent light is given by the following formulae:

$$d\Phi = \Sigma\,(o\cos^2\gamma + e\sin^2\gamma),$$

$$dU = \frac{\pi}{4}\Sigma\,\phi\sin 2\gamma,$$

$$d\Omega = \frac{2\pi}{\lambda}\Sigma\,(u+\tfrac{1}{2}\,du)\,\phi\cos 2\gamma,$$

where

$d\Phi$ is the phase of the vibration along the major axis of the emergent elliptical vibration, with respect to the incident light,
$d\Omega$ is the angle between the major axes of the incident and emergent light,
dU is the ellipticity of the emergent light,
u is the ellipticity of the incident light,
du is the ellipticity of the light emerging from any one plate,
o and e are the times taken by the ordinary and extraordinary vibrations respectively to traverse any one plate,
γ is the angle between one plate and the preceding one, being positive if measured clockwise and negative if anticlockwise,
ϕ is the retardation produced by any one plate,
Σ indicates the sum of all the terms analogous to that written and in which the quantities have the values which apply successively to each of the plates.

For a series of identical plates at random orientation, the incident light being plane-polarized and travelling perpendicular to the plates, ϕ is the same for all plates and

$$\Sigma\sin\gamma = \Sigma\cos 2\gamma = \Sigma\sin 2\gamma = 0,$$
$$\Sigma\cos^2\gamma = \Sigma\sin^2\gamma = \tfrac{1}{2}.$$

Hence

$$d\Phi = \tfrac{1}{2}\Sigma\,(o+e),$$
$$dU = 0,$$
$$d\Omega = 0,$$

i.e. the emergent light is plane polarized in the same direction as the incident light, its velocity in the medium being the average of those of the ordinary and extraordinary vibrations. The direction perpendicular to the plane of the plate is therefore an optic axis.

The same result is obtained by the deduction from the above formulae that, under these conditions, the order of the plates is immaterial; the total effects of the

randomly orientated plates is the same as that of a series in which the plates are arranged in pairs with their axes mutually perpendicular. In a pair of such plates, the vibration which passes through the first plate at the speed of the ordinary vibration passes through the second plate at the speed of the extraordinary vibration and vice versa; if the plates are of equal retardation there is no retardation of one vibration with respect to the other, and the direction perpendicular to the plates is therefore an optic axis.

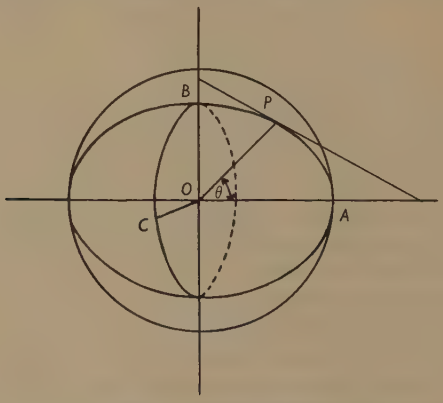

Fig. 1. Velocity ellipse.

The retardation for light passing in any other direction can be calculated from the velocity ellipse (Fig. 1). Let OA represent the optic axis of the first plate, the magnitude $OA=a$ being the velocity of the ordinary wave, and $OB=b$ being the extreme value of the velocity of the extraordinary wave. Then for light travelling in any other direction OP, making an angle (θ) to the optic axis, the ordinary wave travels at velocity a, vibrating perpendicular to the plane of the paper, and the extraordinary wave travels at velocity $OP=p$, vibrating in the plane of the paper. If the optic axis of the next plate lies along OC, then the ordinary wave travels at velocity a, vibrating in the plane of the paper, and the extraordinary wave travels at velocity b, vibrating perpendicular to the plane of the paper.

For vibrations in the plane of the paper, the time taken to traverse the system is, therefore, $l/p+l/a$, where l is the path length in one lamella.

For vibrations perpendicular to the plane of the paper, the time taken to traverse the system is $l/a+l/b$. The path difference per unit path length is $\frac{1}{2}(k/b-k/p)$, and the effective birefringence is $\frac{1}{2}(\mu_b-\mu_p)$. As OP approaches OA, μ_p approaches μ_a and the effective birefringence approaches $\frac{1}{2}(\mu_b-\mu_a)$. The system therefore behaves like a single plate of birefringence $\frac{1}{2}(\mu_b-\mu_a)$.

If the corneal fibres are randomly arranged, then the cornea considered as a 'plate' will have a birefringence half that of the constituent fibres.

The birefringence (ω) of the corneal fibres can then be calculated easily from measurements of the retardation of the cornea for light passing parallel to the axis

of the symmetry. Since the effective birefringence for light passing at an angle α to the optic axis is proportional to $\sin^2\alpha$ (Bear & Schmitt, 1936) the retardation Δ for this light path is given by $\Delta = \frac{1}{2} d\,\omega \sin\alpha \tan\alpha$, where d is the corneal thickness.

Assuming that the refractive index of the cornea is 1·376, the radius of curvature of the anterior surface is 8 mm., and the thickness increases from 0·5 mm., in the centre to 0·9 mm. in the periphery (Steindorff, 1947), the value of angle α, and hence the expected retardation can be calculated for a light path entering the cornea at any point (Table 1). It will be seen that if the birefringence of the corneal fibres is 0·0028, the calculated retardation is in good agreement with the observed retardation.

Table 1. *Theoretical and observed values of retardation ($m\mu$) at various distances from the corneal vertex*

Distance from corneal vertex (mm.)	Thickness of cornea (d) (mm.)	Angle between light path and normal to corneal surface	Theoretical value of retardation ($m\mu$) for coefficient of fibre birefringence of 0·0028	Mean value of observed retardation (Stanworth & Naylor, 1950)
1·0	0·55	6° 58′	11·4	18
2·0	0·60	14° 3′	51	59
3·0	0·65	20° 50′	123	135
4·0	0·70	29° 3′	264	266
5·0	0·775	37° 22′	503	479
6·0	0·85	45° 22′	857	845

SUMMARY

It is shown mathematically that the cornea, in which the fibres are randomly arranged, has a coefficient of birefringence equal to half that of its constituent fibres. Analysis of previously published results indicates that the latter value is 0·0028.

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POLARIZED LIGHT STUDIES OF THE CORNEA

II. THE EFFECT OF INTRA-OCULAR PRESSURE

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A. THE MEASUREMENT OF THE RETARDATION OF THE CORNEA

Preliminary experiments on the change of corneal retardation with changes in intra-ocular pressure have been described elsewhere (Stanworth, 1949, 1950). Theoretical considerations, together with measurements of the retardation of the cornea at different pressures with transmitted light passing in various directions, led to the conclusion that in order to obtain the maximum change in retardation,

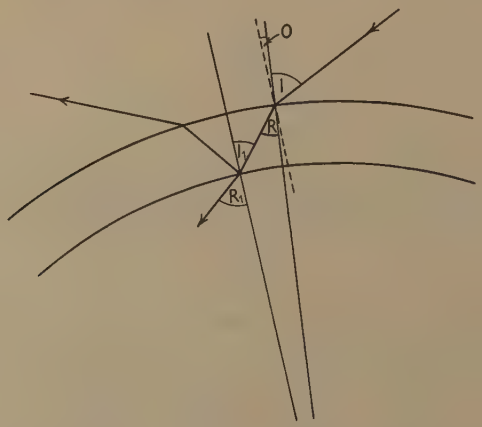

Fig. 2. Light path reflected from posterior corneal surface.

measurements should be made in the central zone of the cornea, preferably with a horizontal light path so that the fibres having the main birefringent effect on the light are the vertical ones. In the intact eye, of course, some intra-ocular surface has to be used as a reflector so that the light can reach the observer. In order to avoid the effect of variations in the depth of the anterior chamber and enable the light to be confined to the central zone, the posterior corneal surface was used, the light path being a symmetrical one in the centre of the cornea (Fig. 1). The theoretical basis of the method is as follows.

(1) *The retardation for obliquely reflected light*

Let the incident plane polarized light be represented by $u_1 = A \cos \omega t$ in the plane of incidence, and $v_1 = B \cos \omega t$ perpendicular to the plane of incidence.

Then using Fresnel's formulae, the light emerging after refraction, reflexion and a second refraction, for a system symmetrical about the point of reflexion (P) on the posterior surface, is given by

$$u_2 = \frac{A \sin 2I \sin 2R}{\sin^2 (I+R) \cos^2 (I-R)} \quad \frac{\tan (I_1 - R_1)}{\tan (I_1 + R_1)} \cos \omega t,$$

$$v_2 = \frac{-B \sin 2I \sin 2R}{\sin^2 (I+R)} \quad \frac{\sin (I_1 - R_1)}{\sin (I_1 + R_1)} \cos (\omega t - \phi),$$

where ϕ is the retardation produced by the cornea of the vertical component compared with the horizontal component. I and R are angles of incidence and refraction for incident light at anterior surface. I_1 and R_1 are angles of incidence and refraction at posterior surface. This is the form of the general expression for an elliptical vibration; that is,

$$\left.\begin{aligned} u &= M \cos \omega t, \\ v &= N \cos (\omega t - \phi). \end{aligned}\right\} \qquad (1)$$

The sign of N/M depends on the relative signs of A and B and the sign of $\tan (I_1 + R_1)$.

From the known curvature and thickness of the cornea the length of the light path and the values of I_1 and R can be calculated for any angle of incidence, the average of the latter being the average angle between the light pathway and the optic axis of the cornea. If then the constants of the elliptically polarized emergent light can be measured, the birefringence of the cornea can be calculated.

(2) *Methods of measurement of the ellipticity of the emergent light*

The most accurate methods of measurement of elliptically polarized light involve the use of some kind of half-shade device; in the present instance, however, the observed field is not uniform, and such devices cannot be used. In addition, the positions of the principal axes of the light vary with the retardation and have to be determined for each measurement. The possible methods that can be used are, therefore, both less accurate and less facile than those in general use. The method adopted was to place a quarter-wave plate with its fast axis along the major axis of the emergent ellipse, thus converting the light to plane-polarized light, the azimuth of which was then measured. The ellipticity (e) of the light is then given by the clockwise angle between the major axis and this azimuth.

The retardation of the cornea (ϕ) is then given by

$$\tan \phi = \frac{\tan 2e}{\sin 2\theta}, \qquad (2)$$

where θ is the azimuth of the major axis of the ellipse.

The sign of $\cos\phi$ is obtained from the formula

$$\cos\phi = \frac{\tan 2\theta}{\tan 2i}, \tag{3}$$

where $\tan i = M/N$. The value of $\phi \pm 2\pi n$ is thus determined.

The disadvantages of the foregoing method are:

(i) In equation (2), used for determining the numerical value of ϕ, a given change in ϕ will lead to only a small change in e when θ is small. The maximum sensitivity will be when $\sin 2\theta = \pm 1$, i.e. $\theta = \frac{1}{4}\pi$ or $\frac{3}{4}\pi$.

(ii) A change in the retardation will, in general, lead to a change in the position of the major axis of the ellipse. The whole procedure for the measurement of the ellipticity should therefore be repeated. Since, however, in the present case, the change in the retardation is relatively small, it was usually sufficient to assume that the position of the axes remained constant, and to take the change in analyser position as a measure of the change in ellipticity. It would obviously be an advantage, however, if the position of the axes remained constant with a change in retardation.

This can be done, and maximum sensitivity obtained, for, if $M = \pm N$, $\tan i = \pm 1$ and $\tan 2i = \pm\infty$. So, from equation (3), $\tan 2\theta = \pm\infty$ for all values of ϕ, i.e. $\theta = \pm\frac{1}{4}\pi$ for all values of ϕ. And, from equation (2) $\tan\phi = \pm\tan 2e$, i.e.

$$\phi = \pm\tan 2e \pm \tfrac{1}{2}n\pi.$$

This arrangement is that used by Goranson & Adams (1933). In view of these considerations, the plane of polarization of the light entering the cornea was always arranged empirically so as to give a value of θ as near as possible to $\pm 45°$.

In practice the desired setting can most easily be obtained by placing the analyser in the diagonal position and rotating the polarizer to give the minimum intensity of the posterior specular reflex. For in these circumstances,

$$I = \tfrac{1}{2} + \tfrac{1}{2}\sin 2i\cos\phi,$$

which has its minimum value when $i = 45°$ or $135°$, depending on the sign of $\cos\phi$.

Since, as described above, a half-shade device cannot be used, the determination of the azimuth of the axes of the ellipse may not always be accurate. To avoid an error from this cause, the quarter-wave plate can be rotated from one side to the other of the estimated position, the least numerical value for the rotation of the analyser—from the position parallel to the axis of the quarter-wave plate to the new minimum position after the insertion of the quarter-wave plate—being a direct measure of the ellipticity of the incident light. That this is the case can be shown as follows:

Let elliptically polarized light with principal axes a and b fall on a quarter-wave plate with its fast axis at an angle α to the major axis of the ellipse, and then on an analyser at an angle e' to the fast axis of the plate.

Then the intensity I of light passed by the analyser is given by

$$I = \cos^2 e'\,(a^2\cos^2\alpha + b^2\sin^2\alpha) + \sin^2 e'\,(b^2\cos^2\alpha + a^2\sin^2\alpha) - ab\sin 2e'.$$

Differentiating I with respect to e' for a constant value of α, we find that, for the maximum value of I,

$$\cos 2\alpha = \frac{2ab}{(b^2 - a^2)\tan 2e''}, \tag{4}$$

where e'' indicates the analyser position for maximum intensity, and e'' is between 0 and $-\frac{1}{2}\pi$ if b/a is positive, e'' is between 0 and $\frac{1}{2}\pi$ if b/a is negative. From equation (4) differentiating e'' with respect to α we find that e'' has a maximum or minimum value when either $b = a$ (the condition for circulary polarized light) or $e'' = 0$ or $\frac{1}{2}\pi$ (which can only be true if the incident light is plane polarized) or $\alpha = 0$ or $\frac{1}{2}\pi$ (which applies to any incident light). In the latter case it can easily be shown that if n is an even integer (i.e. the quarter-wave plate has its fast axis along the major axis of the ellipse) e'' is at its maximum value if b/a is positive.

But it was shown above that e'' is then between 0 and $-\frac{1}{2}\pi$, so e is the minimum numerical value of e'' in a clockwise direction. Similarly if b/a is negative, e is the minimum numerical value of e'' in an anti-clockwise direction.

These relationships are reversed if n is an odd integer, the quarter wave plate having its slow axis along the major axis of the original ellipse.

Results

The Fresnel formulae used are only approximate, so it was considered necessary to verify the method experimentally. A piece of adhesive cellophane tape was fixed on a glass slide, and its retardation measured for normal incidence by transmitted light. The slide was then rotated, first about an axis containing the optic axis of the cellophane tape, and then about an axis at right angles to this, the retardation being measured for varying angles of incidence. The retardation was then measured by light reflected from the back surface of the glass slide; the maximum error was nowhere greater than 6 %. The experimental results with the cat cornea also showed that the method is reasonably accurate; the retardation of the cornea in a pressure chamber (Stanworth & Naylor, 1950) was measured under constant conditions but with different positions of the polarizer. The calculated retardation varied by only ± 10 mμ from the arithmetical mean.

The preliminary results (Stanworth, 1950) obtained by reflected light from a cornea suspended in a pressure chamber correspond to a birefringence of the corneal fibres of 0·0037 (for light of wave-length 540 mμ), almost all the results falling between 0·0030 and 0·0045. The change in birefringence when the pressure behind the cornea was raised from 10 to 40 mm. Hg averaged 0·00012, i.e. about 3% of the total. The rate of increase in birefringence decreased at higher pressures, but this had little if any effect below a pressure of about 30 mm. Hg, i.e. over the physiological range.

The experiments were repeated with the whole eye suspended by sutures through the episclera, the pressure being varied through a needle inserted through the optic nerve and past the dislocated lens. In this case the change in birefringence for the above change in pressure was about 7 % of the total. It appears, then, that

by far the greater part of the double refraction of the cornea is determined by the structure of the corneal fibre and only a relatively small proportion is due to stress.

B. THE MEASUREMENT OF CHANGES IN RETARDATION BY PHOTOGRAPHY

Measurement of retardation by density of a photographic image can be very sensitive (Swann & Mitchison, 1950), and was used in the present case in an attempt to make the method sufficiently easy to envisage its application to the human subject.

For the light leaving the cornea, the intensity I passed by an analyser at angle γ to the horizontal is

$$I = M^2 \cos^2 \gamma \text{ and } N^2 \sin^2 \gamma + MN \sin 2\gamma \cos \phi.$$

To obtain the greatest rate of change of intensity with changes in retardation, MN and sin 2γ should have their maximum values, i.e. $M = N$, and $\gamma = 45°$ or $135°$; in these circumstances, $I = \sin^2 \frac{1}{2}\phi$, and the polarizer is set as before.

In practice it was not convenient to set the polarizer in this manner, since the anterior specular reflex was not then at minimum intensity and tended to overlap the posterior reflex. The polarizer was set to give minimum intensity of the anterior reflex. Under these circumstances $I = \frac{1}{2} \pm MN \cos \phi$, and the rate of change of intensity is not at its maximum value.

Whatever the polarizer setting, the maximum change in intensity for a given change in ϕ will occur when $\phi = \frac{1}{2}\pi \pm n\pi$. If the phase difference is between 0 and 180°, the intensity should increase, but if it is between 180 and 360°, it should decrease, with rising pressure. If it is slightly less than 180°, the slight initial increase in intensity will not produce any appreciable increase in density, and only the subsequent fall will be seen. If the initial phase difference is slightly lower still, a preliminary rise will be followed by a fall. The reverse will occur with an initial phase difference slightly less than 360°.

These predictions were confirmed by measurement, by an S.E.I. densitometer, of 500 photographs taken at different pressures, using the cornea suspended as before and fast orthochromatic film (Kodak Ortho. X), at $\frac{1}{2}$ sec. exposures, in a specially constructed fixed focus miniature camera giving a magnification of $\times \frac{3}{4}$. Corneae with phase differences from 340 to 360° and 0 to 160° showed an average increase in image density, with increasing pressure, of 0·13; those with a phase difference 180–330° showed an average fall in density of 0·15. Corneae with phase differences of 168, 100 and 330° showed a biphasic response.

These changes in density are sufficiently marked to make it possible to envisage an instrument which, although not recording the intra-ocular pressure directly, would nevertheless measure changes in its value in the human eye with a considerable degree of accuracy, without touching the eye.

SUMMARY

A method is described of measuring the birefringence of the intact cornea using light reflected obliquely from its posterior surface. The value obtained (0·0037) is in reasonable agreement with the results obtained by using transmitted light and from corneal sections. The change in birefringence with an increase in intraocular pressure from 10 to 40 mm. Hg is about 7 % of this. The change in birefringence was also assessed by changes in the density of the photographic image of the reflected light from the surface, and this affords a possible way in which the intraocular pressure could be measured in the human subject without touching the eye.

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ON CERTAIN PROPERTIES OF THE FLIGHT MUSCLES OF THE ORTHOPTERA

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(With Plate 9)

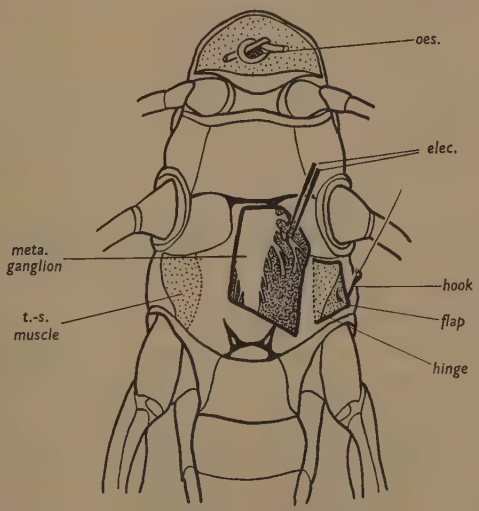
The physiological characteristics of the wing musculature of insects have been but little investigated. Solf (1931) has given a brief account of the responses of the dorsal longitudinal muscles of *Gryllotalpa vulgaris* (L.) to direct electrical stimulation. A more extensive study has been made by Heidermanns (1931) of the responses to direct electrical stimulation of the wing muscles of *Aeschna coerulea* (Ström). The latter author found that at a stimulation frequency comparable with that of the normal wing beat of the dragonfly, the muscles were in a partial tetanus. He concluded that this was their normal condition in flight. More recently, Pringle (1949) has found that potentials recorded from the wing muscles of *Calliphora* occur at a lower frequency than the wing movements. He concluded that these flight muscles were highly specialized, and that impulses from the nervous system activated rather than excited the muscles. Similar observations have been made by Roeder (1950, 1951) on *Vespa*, *Lucilia* and *Tabanus*. In *Periplaneta* and the moth *Agrotis*, however, Roeder recorded a single muscle potential for each cycle of the wing beat. He concludes that in the latter two species the wing-beat frequency was such as to permit a conventional neuromuscular system to move the wings.

In 1947, Voskresenskaya published an account of the responses of the tergosternal and associated vertical direct-flight muscles of *Locusta migratoria* L. when either the nerve supplying the tergo-sternal muscle or the metathoracic ganglion was stimulated electrically. The nerve supply to these muscles was in all cases left attached to the ganglion. The muscular responses were recorded from the movements of the hind wing. With this preparation she was able to show that at stimulation frequencies below 20 cyc./sec. the wing muscles respond with discrete contractions. At higher frequencies the response of the muscle depends upon the intensity of stimulation; at low intensities the muscles respond with discrete contractions at 12–18 beats/sec., but at higher intensities a clonus or a smooth tetanus can be obtained. It will be appreciated that this preparation is complicated both by the large number of muscles concerned in the movement of the wing and by the connexion of the nerves to the metathoracic ganglion. It is, moreover, not clear whether in removing the dorsal longitudinal muscles the sensory nerves from the basalar and subalar regions which join with this motor nerve were cut.

In ignorance of Voskresenskaya's investigations we have performed essentially similar experiments, also using *L. migratoria*. These are briefly described below. The preparation we have employed records the movement of the tergo-sternal muscle only (muscle 113; Snodgrass, 1929), although the nerve stimulated supplies muscles 118, 125, 126, 127 and 128, some of which are concerned in the movement of the wing. The results we have obtained agree well with those of Voskresenskaya, but the mechanical records alone are insufficient to give a definite interpretation of the effects observed. We have now had the opportunity to make electrical recordings from these muscles which permit a decision between the various interpretations which can be offered. The second half of this paper is concerned with these recordings.

KYMOGRAPHIC RECORDINGS

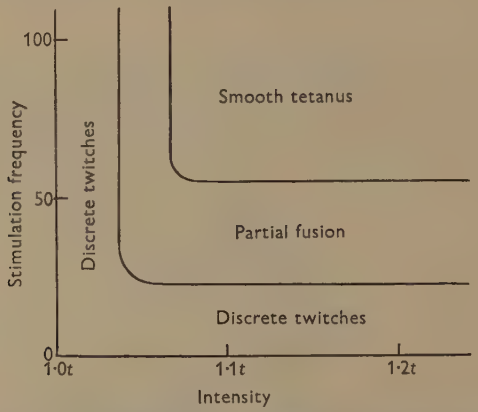
The preparation used was made as follows. The animal was decapitated. An approximately rectangular portion of the metathoracic sternite was removed to expose the ganglion. The nerve trunk supplying the tergo-sternal and other muscles was crushed at its point of emergence from the ganglion, and all other



Text-fig. 1. Ventral view of *Locusta* prepared for recording of tergo-sternal muscle movement. *elec.* electrodes. The *flap* of exoskeleton with an inserted *hook* and mesial *hinge* line may be seen. *meta. ganglion*, metathoracic ganglion. *oes.* cut and ligatured oesophagus. *t.-s. muscle*, origin of the tergo-sternal muscle.

homolateral nerves severed close to the ganglion. Care was taken not to damage the ventral air sacs, tracheae or salivary glands. Incisions were then made through the lateral extensions of the metathoracic basisternite (Text-fig. 1). These incisions freed three sides of a small flap of exoskeleton which carries only the origin of the tergo-sternal muscle. The remaining muscles innervated by the nerve take their

origins more laterally. The flap remained attached along its mesial side which acted as a hinge. A small metal hook was inserted into the free edge of this flap, clear of the muscle attachment; a thread from the hook was attached to a light isometric lever. The flap was reflected outward from the body so that the muscle was under a slight initial tension. The nerve was stimulated with a square wave oscillator (Leisegang & Ripley, 1949) using platinum electrodes. The normal period of stimulation was 5 sec.; the preparation was allowed 1 min. rest after each stimulation. The preparation continued to make respiratory movements and the neuromuscular system being studied remained excitable for 3–6 hr. All experiments were conducted at 28° C.

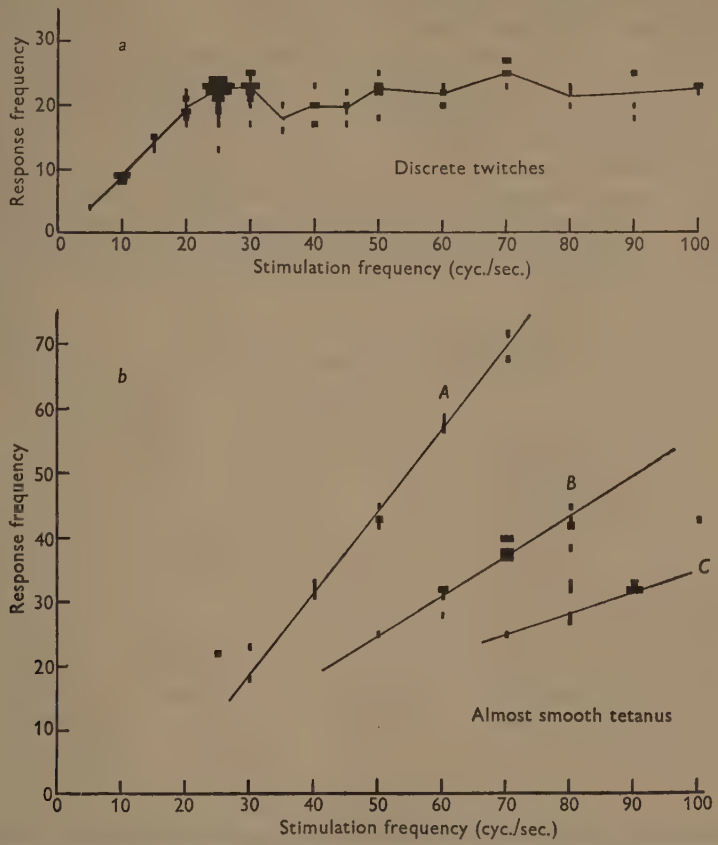


Text-fig. 2. Diagrammatic representation of the relationship between frequency and intensity of stimulation and the character of the muscular response. t = threshold voltage.

The general form of the responses obtained at different frequencies and intensities of stimulation is summarized diagrammatically in Text-fig. 2. At low intensities of stimulation the muscle responds with a series of discrete twitches regardless of the frequency of stimulation. At moderate intensities a partial fusion of the response is obtained with all frequencies of 25 cyc./sec. and greater, but no smooth tetanus is developed. At still higher intensities partial fusion is observed between 25 and 60 cyc./sec.; above this frequency the muscle responds with a smooth tetanus.

A detailed study of the response frequency of the preparation shows that at low frequencies of stimulation the discrete twitches closely follow the stimulation frequency; the slight differences between the stimulation and response frequency are probably due to errors in calibration. At frequencies above 20 cyc./sec. and at intensities producing discrete twitches only the frequency of response becomes remarkably constant. This may be seen in Text-fig. 3*a*, in which the response frequency is plotted against stimulation frequency. The mean frequency for all such cases recorded between stimulation frequencies of 25 and 100 cyc./sec. is

22·4 ± 0·25 beats/sec. This appears to accord well with the wing-beat frequency of 20 beats/sec. for intact locusts reported by Kennedy, Ainsworth & Toms (1948).



Text-fig. 3. Relationship between the stimulation and response frequencies of the tergo-sternal muscle. The width of the blocks indicates the number of observations; for example, at a stimulation frequency of 10 cyc./sec., five responses were recorded at 9 cyc./sec. and three at 8 cyc./sec. *a*, responses are discrete twitches. The continuous line joins the means of the response frequencies at different stimulation frequencies. *b*, responses are almost smooth tetanus. *A*, *B* and *C* are explained in the text.

A very different picture is obtained from responses in which the tetanus is almost smooth (Text-fig. 3*b*). Here there are suggestions of various modes of response tentatively indicated by the three lines *A*, *B* and *C*.

These results are in complete agreement with the findings of Voskresenskaya. We are able further to confirm her statement that the tergo-sternal muscles require far higher frequencies of stimulation than do those of the legs to produce a partial

fusion of contractions and a smooth tetanus. Results obtained from a study of different metathoracic leg muscles stimulated by way of the main nerve trunk are shown for comparison in Table 1.

There appear to be two possible interpretations of the results which we have obtained from the tergo-sternal muscle. The first is that there exists in these muscles a mechanism somewhat similar to that found by Pringle in *Calliphora*. The relative constancy of the response frequency found in Text-fig. 3*a* would then be regarded as due to an inherent myogenic rhythm. The smooth tetanus obtained at higher intensities of stimulation could be attributed to a second system of 'tonic' fibres in the nerve trunk with a higher threshold than those stimulating the muscle to contract with discrete twitches. Complicating assumptions would have to be introduced to explain the type of results which are shown in Text-fig. 3*b*.

Table 1. *Frequencies at which fusion of contractions and smooth tetanus first appear in different muscles of* Locusta

Muscle	Fusion (cyc./sec.)	Smooth tetanus (cyc./sec.)
Depressor tibiae	8	25
Levator tibiae	19	25
Depressor tarsi	11	20
Levator tarsi	16	20
Tergo-sternal	25	60

Note. The first four values refer to the muscles of the metathoracic leg of *phasus gegraria*.

The alternative explanation lies in the assumption that while the muscle itself has the normal properties of striped muscle, the nerve supplying it is characterized by the fact that following stimulation there is a long period of subnormal excitability. At low intensities and high frequencies of stimulation the stimuli will be counted down by some approximate submultiple of the stimulation frequency. Increasing intensity of stimulation will result in more nerve impulses reaching the muscle, which will pass through varying degrees of fusion to a smooth tetanus when both stimulation frequency and intensity are high enough. Text-fig. 3*a* then merely reflects the fact that a frequency of 20–25 beats/sec. is about the fastest frequency at which the muscle can contract without the development of residual tension. The variations between the mean frequency of response at different stimulation frequencies may be regarded as a product of this counting down. This explanation also allows an interpretation of Text-fig. 3*b*. The line *A* represents a response from the muscle to almost every shock, *B* to almost every second shock and *C* to almost every third.

Electrical recordings from the muscles should permit a distinction between these alternatives. If the first interpretation is correct the contractions of the muscles may be expected to be independent of the stimulating shocks at high frequencies and low intensities of stimulation; if the latter, such contractions as occur should follow particular stimuli.

ELECTRICAL RECORDINGS

The responses of the muscles were recorded with fine platinum electrodes inserted directly into the muscles being studied. The responses were amplified with a Grass pre-amplifier and the amplifiers of a Cossor oscillograph. The relevant nerve was stimulated with a simple condenser discharge circuit.

Using a preparation essentially similar to that in the preceding section a study was made of the responses of the tergo-sternal muscles and also of the direct depressor-extensor of the hind wing (muscle 129) which lies beneath the epimeron of the metathorax. To be able directly to observe the behaviour of the indirect flight muscles when stimulated, a simple preparation of the thorax cut parasagittally was used. The musculature of one side was removed in this manner, while the metathoracic ganglion and the musculature of the opposite side remained in position. By suitable dissection the relevant nerves could be exposed and direct observations made on the musculature. Electrical recordings from the dorsal longitudinal muscle (muscle 112) were also made with this preparation. Using *Periplaneta americana* (L.) a few recordings were made from a muscle which runs from the base of the wing to the meron of the coxa (muscle 169; Carbonell, 1947); this muscle is homologous with the depressor extensor of the hind wing of the locust. All experiments were performed at room temperature (*c.* 18° C.).

The results obtained clearly indicate that the various muscles studied respond to nervous stimulation, and there is no suggestion of any myogenic rhythm. Pl. 9 shows a series of records from the dorsal longitudinal muscle of a single preparation stimulated at 62 cyc./sec. The three records, *a*, *b* and *c*, were taken at different intensities of stimulation, and it can be seen that the muscle is responding to every fourth, every third and every second shock as the intensity of stimulation is increased. Such regularity of response as is shown by these records is not always found. Text-fig. 4*d* shows a record from the tergo-sternal muscles at the same stimulation frequency. The very irregular character of the response, producing an average frequency of 18 contractions/sec., can be seen. It would thus appear that the explanation of the effects described in the previous section lies in a prolonged period of subnormal excitability in the nerve.

From a study of the minimal response frequency an estimate can be made of the duration of this period of subnormal excitability. For the two indirect muscles and the depressor extensor of the locust a value of about 75 msec. is found. For the depressor extensor of the cockroach a smaller value of 25–30 msec. appears. The long period of subnormal excitability of the nerves of *Locusta* corresponds to a response frequency of 12–14 contractions/sec. This condition will only be found when the stimulus intensity is just supra-liminal, and in practice with stimulation frequencies within the range of 35–65 cyc./sec. the response frequency usually found at low stimulation intensities is 18–24. This agrees well with the results shown in Text-fig. 3*a*, using kymographic recording.

Although in the preparation of the tergo-sternal muscle which we have employed to record mechanical contractions there is every reason to believe that the pre-

paration records the movement of this muscle alone, it seemed desirable to check by visual observation that the indirect flight muscles did contract tetanically at high intensities and frequencies of stimulation of their nerve supply and thus to eliminate finally the possibility that the records of a smooth tetanus might be due to the tonic contractions of some direct muscles. Direct observations of the behaviour of the two sets of indirect muscles were made using the parasagittally cut thorax. Both muscles were observed to contract into a smooth tetanus when their nerves were strongly stimulated at about 60 cyc./sec.

CONCLUSION

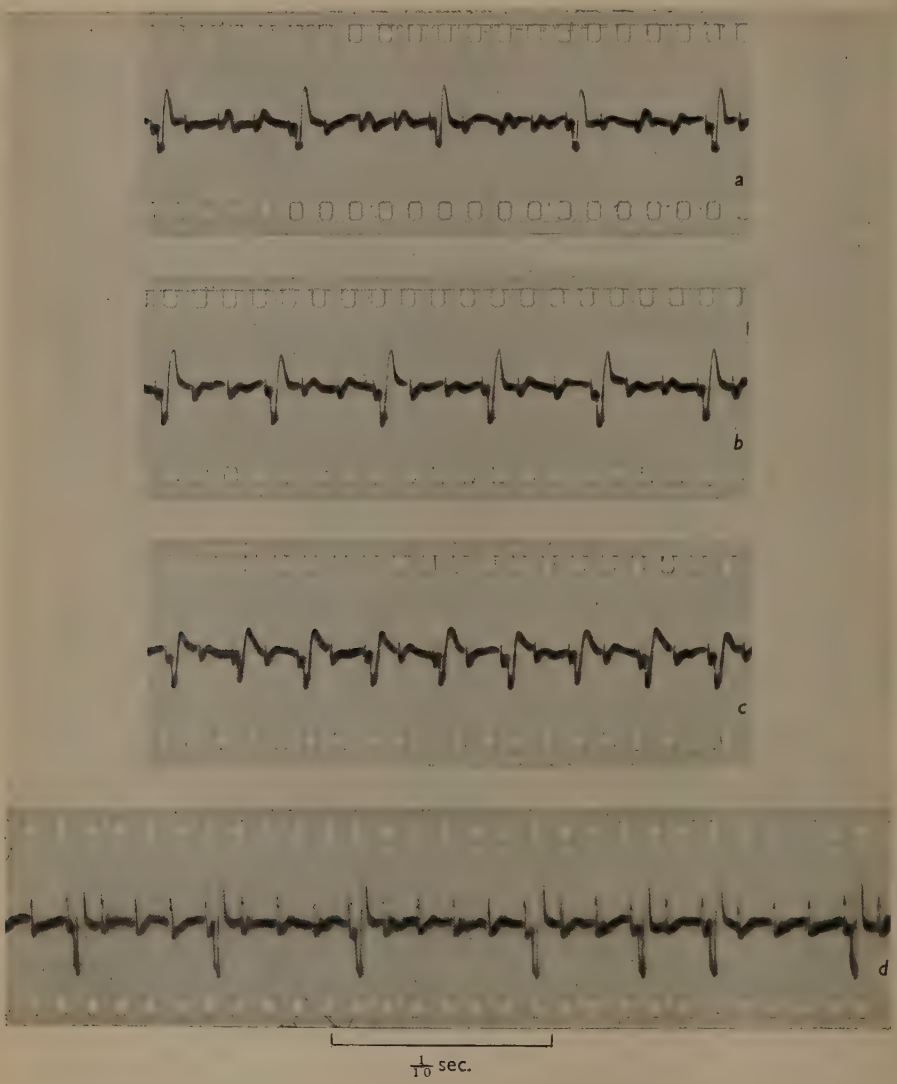
From these results it first appears that the indirect flight muscle of the locust thorax can contract without fusion at a frequency comparable to the normal wing beat of the animal. It is noteworthy that in the leg muscles of *Periplaneta* fusion of contractions commences at a higher frequency than in those of *Locusta*; the frequency characteristics found in *Periplaneta* resemble those which we have found in the tergo-sternal muscles of *Locusta*. Thus Pringle (1939), using the extensor tibiae, found that with the quick fibre fusion of contractions started at about 30 cyc./sec. and a smooth tetanus developed above 70 cyc./sec. The same effect, but still more pronounced, has been found by Roeder & Weiant (1950) with the tergal muscles of the trochantin of *Periplaneta* where fusion of contractions does not begin until the motor nerve is stimulated at a frequency above 40 cyc./sec., and complete fusion has still not been attained at 100 cyc./sec. These results may be compared with the values found for the leg muscles of *Locusta* shown in Table 1. They suggest that the performance of the tergo-sternal muscles may well be typical of orthopteran skeletal muscle, but that the muscles of the hind leg of *Locusta* are specialized. This may possibly be a reflexion of the specialization of the metathoracic limb of *Locusta* for jumping. It is, however, clear that there is no need to assume that in the locust the flight muscles are in a state of partial tetanus during flight, as has been suggested for the flight muscles of *Aeschna* by Heidermanns (1931).

The second result is that there is no evidence to be found of any specialization leading to an inherent rhythm of contraction in the indirect flight muscles. The effects obtained which suggested such a possibility have been shown to be due to a prolonged subnormal excitability of the nerve fibres after the passage of an impulse; this is possibly comparable with that found by Grundfest (1939) in mammalian *B* fibres. The exact character of this effect can, however, only be decided by further study.

SUMMARY

1. Kymographic recordings have been made of the movements of the tergo-sternal muscles of *Locusta migratoria* when the motor nerve is stimulated at different frequencies and intensities.

2. At low intensities of stimulation the muscles respond with discrete twitches regardless of the frequency of stimulation. At high intensities and frequencies the



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muscles contract with a smooth tetanus. These findings confirm the earlier observations of Voskresenskaya (1947).

3. Electrical recordings from the tergo-sternal (muscle 113), dorsal longitudinal (muscle 112) and depressor extensor (muscle 129) of the locust wing, as well as from the muscle corresponding to the latter in the cockroach, show that the discrete twitches obtained at high-frequency and low-intensity stimulation arise from a long period of subnormal excitability of the nerve and are not to be attributed to any inherent rhythm of contraction in the muscles.

This work was made possible by a grant from the South African Council for Scientific and Industrial Research, to whom our thanks are due; during part of this work one of us (S.H.R.) held a bursary from the Council. We should like to record further our gratitude to the Chief Locust Officer of the South African Department of Agriculture for his generosity in keeping us supplied with locusts.

One of us (D.W.E.) wishes further to record his thanks to Prof. James Gray for his kindness in according him the hospitality of the Zoological Department in Cambridge and to J. W. S. Pringle for his advice and assistance.

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EXPLANATION OF PLATE 9

Electrical responses from the indirect flight muscles of *Locusta migratoria*. The stimulus artifact can be seen as a fine line. The muscle response is a well-defined diphasic break. *a*, *b* and *c* are records from the dorsal longitudinal muscles at three intensities. *d* is a record from the tergo-sternal muscle.

A NEW METHOD OF MEASURING THE ACTIVITY OF SPERMATOZOA

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(*Received* 28 *October* 1952)

INTRODUCTION

The problem of measuring the speeds at which spermatozoa swim presents many difficulties. If the suspension is sufficiently dilute, measurements can be made by the 'dark-ground track' method (Rothschild & Swann, 1949; Rothschild, 1951). The spermatozoa are illuminated with a dark-ground condenser, and a photographic plate is exposed for a known time above the suspension. As the head of the spermatozoon scatters more light than the tail, a track is formed on the photographic plate. The length of the track divided by the exposure time gives the speed of the spermatozoon. This method cannot be used when the sperm suspension is of normal density, as the spermatozoa in the field scatter too much light for photography. Unfortunately, the dilution necessary to achieve successful results is such that the spermatozoa may undergo physiological changes, often of a pathological nature, immediately after dilution. These changes constitute the well-known dilution effect. Apart from the dark-ground track method, attempts have been made to measure by eye the speeds at which individual spermatozoa swim. This naturally gives no information about the speeds at which representative spermatozoa swim, nor about the important property of a sperm suspension, its mean or average speed. Grave & Downing (1928) measured the time that *some* spermatozoa in a suspension took to swim along a capillary tube of known length. Such observations tell one little about the speeds at which spermatozoa swim—even the ones under observation; for it was assumed, without any justification, that the spermatozoa swam straight along the capillary tubes. The problem of measuring sperm speeds has proved so intractable that many workers concerned with this subject have contented themselves with examining a suspension under the microscope and assigning marks to it according to its activity, assessed by eye. The reader may ask why one cannot take a ciné-film of a sperm suspension and subsequently project the film frame by frame, plotting the positions of individual spermatozoa. The lines connecting the consecutive positions of the spermatozoa can clearly be used to calculate their speeds and therefore the mean speed of the suspension. There are two objections to this method, though it has been used (Rothschild, 1950). First, it is extremely time-consuming; secondly, unless high film speeds are used, it may not be possible to identify one particular spermatozoon on successive frames. This point is illustrated in Fig. 1. Errors due to this uncertainty will falsify estimates of

mean speed and distort the distribution of sperm speeds, if this is of interest. It also creates serious problems for the person plotting the positions of the spermatozoa, as difficult decisions have continually to be made. The uncertainty can of course be obviated by using sufficiently high film speeds, but this increases the labour of plotting and the cost of the experiment.

The object of this paper is to describe a new and quantitative method of estimating the mean speed of a sperm suspension of any density, the estimate having a known precision.* The method is based on the principle known as probability-after-effect, which is described in some detail in Chandrasekhar's review, 'Stochastic problems in physics and astronomy' (1943). Little attention has hitherto

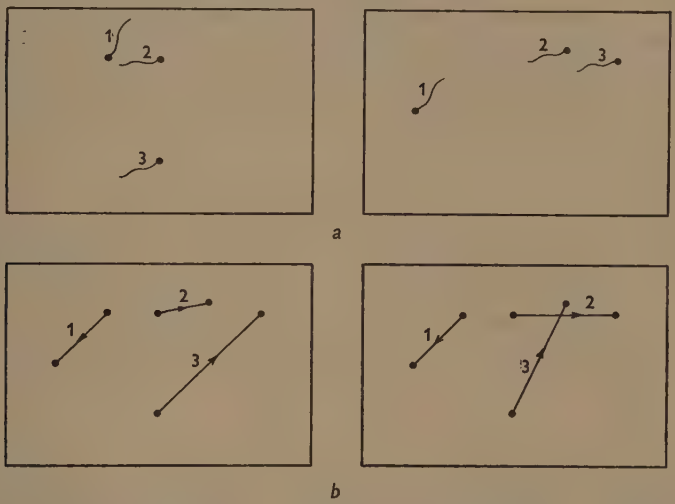

Fig. 1. *a*, positions of three spermatozoa on consecutive cinematograph frames. *b*, alternative routes by which two of the three spermatozoa could get from their positions on the first frame to their positions on the second. There is no chance of a mistake in the case of sperm 1 as it is swimming in the opposite direction to sperms 2 and 3.

been paid to the precision of the method, without which it is of limited interest. The principle of probability-after-effect can be roughly described as follows. Suppose we examine two suspensions of particles or organisms, A and B, and that the particles in A are 'frozen' so that they do not move. The average speed of the particles in A is therefore zero. The particles in B are not 'frozen' and move about. If a small region in A is observed at times t_1 and t_2, the number of particles in that region will be the same. This will not be the case in a small region in B, because some particles which were outside the region at t_1 may be inside at t_2, while some which were inside the region at t_1 may be outside at t_2. The numbers

* A note on the method has been published in *Nature* (Rothschild, 1953).

entering and leaving the region will not necessarily be the same. We can say that in the case of system A, the number of particles in the region at t_1 is completely 'correlated' with the number in the region at t_2. In the case of system B, the number of particles in the region at t_1 will be *partly* correlated with the number in the region at t_2, because particles have entered and left the region in the time interval (t_1, t_2). If the time interval between observations were made sufficiently long, the correlation between the numbers would be zero. The fluctuations with time in the number of particles in the region under consideration is clearly some function of the speeds at which the particles move (actually the mean speed of the particles). Consequently, the mean speed should in principle be determinable from the observed time fluctuations in the number of particles.

When estimates of mean speed are made by the probability-after-effect method of analysis, the experimental procedure is to place a suspension of spermatozoa on a microscope slide and photograph it at given time intervals. The number of spermatozoa in a region of known area is then counted, on each photograph. The mean speed of the suspension can then be estimated by means of a few simple calculations.

THEORY

Random movements of spermatozoa. In its simplest form, the theory requires that the directions of movement of the spermatozoa should be random. If this is so, the spatial distribution of spermatozoa in the suspension will also be random. It can be shown (Appendix, 1) that in such circumstances, the number of spermatozoa in a small region within the suspension will fluctuate Poisson-wise, provided that the sperm counts are made at sufficiently long time intervals (see later). In other words,

$$\Pr\{x\} = \frac{1}{x!}\bar{n}^{x} e^{-\bar{n}} \quad (x = 0, 1, 2, 3, \ldots), \tag{1}$$

where $\Pr\{x\}$ is the probability of there being x spermatozoa in the region and $\bar{n}$ is the average number of spermatozoa in the region.

Probability-after-effect. Let the number of spermatozoa, n, in a region of known area be $n_1, n_2, n_3, \ldots$ in successive photographs taken at equal time intervals $t_1, t_2, t_3, \ldots$. Let $|n_2 - n_1| = \delta_1$, $|n_3 - n_2| = \delta_2$, ... and $t_{i+1} - t_i = \tau$. Then it can be shown, Appendix, 2, that

$$E(\delta^2) = 2E(n)(1 - e^{-\lambda\tau}), \tag{2}$$

where $E(\delta^2)$ = the expected value of δ^2, $E(n)$ = the expected value of n, and λ is a parameter which includes the average or mean speed, $\bar{c}$, of the sperm suspension.

In practice, equ. (2) becomes

$$\overline{\delta^2} = 2\bar{n}(1 - e^{-\lambda\tau}), \tag{2.1}$$

where $\overline{\delta^2}$ = the estimated average value of δ^2, $\bar{n}$ = the estimated average value of n, and λ includes the estimated value of $\bar{c}$. By use of the concept of collision

frequency (Rothschild, 1952), it can be shown (Appendix, 4), that if the region under examination is a rectangle with sides x' and y', a square with side x', or a circle of radius r,

$$\left.\begin{aligned}\lambda &= \frac{2\bar{c}(x'+y')}{\pi x' y'},\\ \lambda &= \frac{4\bar{c}}{\pi x'},\\ \lambda &= \frac{2\bar{c}}{\pi r}.\end{aligned}\right\} \tag{3}$$

These relationships are derived in Appendix, 3.

Correlation. In the section entitled 'Random movements of spermatozoa', reference was made to the numbers of spermatozoa in a small region being distributed Poisson-wise, *provided counts are made at sufficiently long time intervals.* Suppose that consecutive counts have a short time interval between them so that the spermatozoa move a small distance in that time interval. Assume, for example, that at t_1, the number of spermatozoa in a square with side 100μ is counted, and at t_2, 1/24 sec. later, the number in the same square is counted again. Assuming a mean sperm speed of 120μ/sec., each spermatozoon will on the average move 5μ in 1/24 sec. As a result, few of the spermatozoa within the square at t_1 will have swum out by t_2, and few will have swum into the square in the interval (t_1, t_2). In such a case the numbers will not fluctuate Poisson-wise, and the joint distribution of consecutive observations will be a bivariate correlated Poisson distribution in which

$$\text{corr}\,(n_1, n_2) = e^{-\lambda\tau}, \tag{4}$$

where corr (n_1, n_2) is the correlation coefficient of n_1 and n_2 in this system. The derivation of equ. (4) is given in Appendix, 5. This concept is important as we shall be concerned with the best value of corr (n_1, n_2), i.e. the value which maximizes the precision of estimates of $\bar{c}$.

Precision of estimates of $\bar{c}$. Denoting $e^{-\lambda\tau}$ by x and the number of sperm counts made on different frames by k, the variance of $\bar{c}$, $V(\bar{c})$, is given by

$$V(\bar{c}) = \frac{\bar{c}^2}{k\bar{n}(x \log_e x)^2}\left\{\frac{\bar{n}(1-x)^2(3+x)}{(1+x)} + x(1-x)\right\}. \tag{5}$$

The derivation of equ. (5) is very complicated, and so as not to overweight this paper with mathematics, it will be published elsewhere.* This equation enables one to calculate the value of τ, the time interval between photographs, which gives maximum precision for any particular k or $\bar{n}$ in the region under examination. As regards the latter, if the area of the region is reduced, the average number of

* In deriving equ. (5), the probability-generating function of n_2 given n_1,

$$\exp -\{n(1-s)(1-x)\}\{1-(1-s)x\}^{n_1},$$

is used. From it, the relationship $E(n_2 \mid n_1) = n(1-x) + n_1 x$ follows (see Appendix, 5).

spermatozoa in it will also be reduced, and vice versa. Fig. 2 shows a set of curves derived from equ. (5). If this equation is written

$$V(\bar{c}) \div \frac{\bar{c}^2}{k}\{f(\bar{n}, x)\}, \qquad (5.1)$$

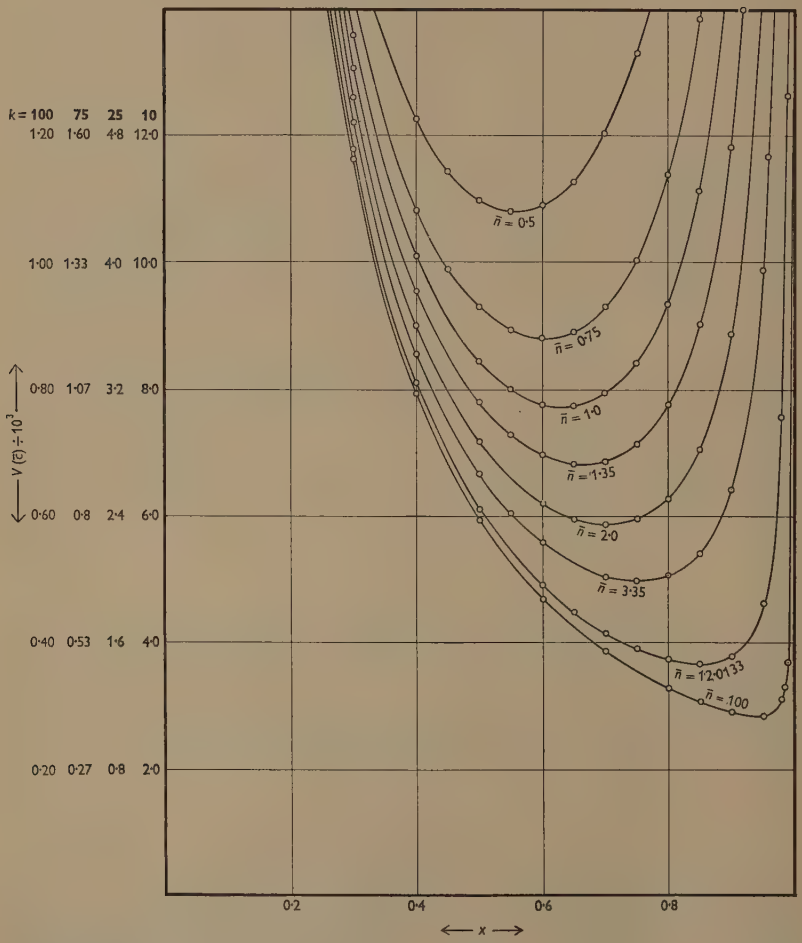

Fig. 2. Relationships between $V(\bar{c})$, the variance of an estimate of $\bar{c}$ (assumed to be 110·5 μ/sec.) and $x = e^{-\lambda\tau}$, for different values of $\bar{n}$, the average number of spermatozoa counted. Four $V(\bar{c})$ scales are given, appropriate to the cases where $k = 10$, 25, 75 and 100.

it becomes clear that, with an appropriate change of ordinate scale, the curves in Fig. 2 can be used with any values of k. Four ordinate scales, relevant to

$k=10$, 25, 75 and 100, are given in Fig. 2. In this case, for reasons that will be apparent later, $\bar{c}$ was taken as 110·5 μ/sec. By laying a ruler horizontally across the curves in Fig. 2, at some particular value of $V(\bar{c})$, experimentally convenient values of $\bar{n}$, k and $\tau(=-1/\lambda \log_e x)$, corresponding to that variance, can be selected. For example, if a variance of 1000 is required, $k=100$, $\bar{n}=100$ and $x=0{\cdot}337$ or 0·999; or $k=100$, $\bar{n}=2$ and $x=0{\cdot}365$ or 0·915. We can, in fact, draw a system of iso-variance curves or 'isologs', from which the values of $\bar{n}$, k and τ corresponding to any particular variance can be readily obtained. These curves are given in Fig. 7, which it is convenient to put in the section entitled 'Practical considerations'. The curves relating $V(\bar{c})$ and τ at different levels of $\bar{n}$ are also instructive and are given in Fig. 3.

Loss of information. Previous workers on probability-after-effect do not seem to have realized that if one only observes the number fluctuations in successive *pairs* of observations, a significant amount of information is lost. The point is best made by means of a diagram. Fig. 4*a* refers to the case where a comparison is only made between successive pairs of counts. But a comparison of the numbers in frame 1 and those in frames 3, 4, 5, ... (Fig. 4*b*) provides further information, using the method of Maximum Likelihood. There is yet another source of loss of information. Both in Fig. 4*a* and *b*, comparisons are made between the numbers at different times in the *same* region in the suspension. But apart from the temporal relationships to which reference has been made, spatio-temporal relationships also exist; these can provide further information, a point which is made in Fig. 4*c*. The extraction of this additional information presents formidable mathematical difficulties. I hope to examine these questions later, with Dr H. Ruben.

EXPERIMENTAL PROCEDURE

Bull semen was diluted 1/4 with phosphate buffer (0·6 g. KH_2PO_4 and 6 g. Na_2HPO_4 in 300 ml. water) containing 5 mg. fructose/ml. A drop of this diluted semen was placed on a microscope slide and a no. 1 cover-slip ($\frac{7}{8}\times\frac{7}{8}$ in.) was very gently placed on top of the drop. The size of the drop was so adjusted that after the cover-slip had been put on top of it, the drop spread just to the edges of the largest circle which could be inscribed on the cover-slip, without any pressure being applied. In these circumstances the spermatozoa swim actively in a particular lamina of fluid between the lower surface of the cover-slip and the top surface of the microscope slide.* This point is important, as the analysis involves considerations similar to those used in the kinetic theory of gases, but in two, not three, dimensions. If, therefore, the field examined under the microscope were a cylinder rather than a disk, the theoretical foundation on which the method is based would be wrong, for reasons shown pictorially in Fig. 5. This does not mean that a three-dimensional system cannot be used in other types of experiment, but that the formulae used in this paper would have to be altered.†

* The fact that spermatozoa swim 'best' in particular laminae in the suspension is an interesting phenomenon for which, at present, there does not seem to be any obvious explanation.

† In the case where the region under examination is a cylinder of height h as in Fig. 5*b*, the only alteration is that equ. (3) becomes $\lambda=\frac{\bar{c}}{2}\left(\frac{1}{\tau}+\frac{1}{h}\right)$.

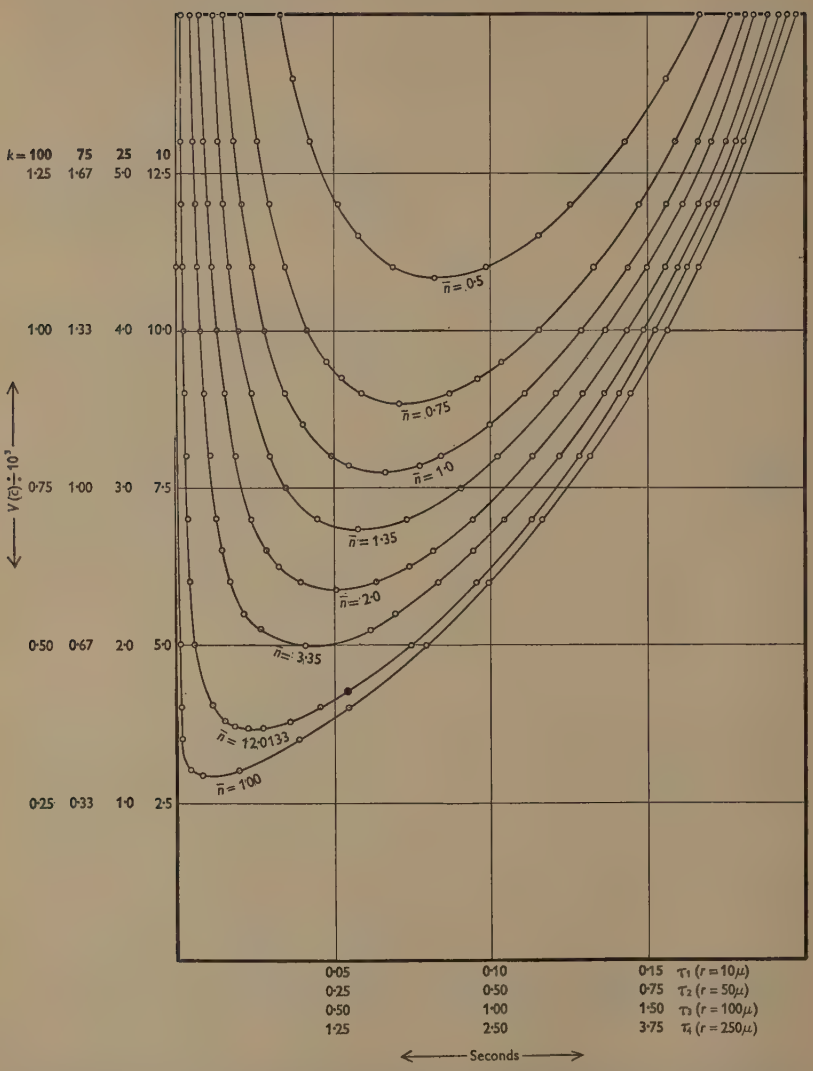

Fig. 3. Relationships between $V(\bar{c})$ and $\tau(=-1/\lambda \log_e x)$ at different levels of $\bar{n}$. Four $V(\bar{c})$ scales, appropriate to $k = 10$, 25, 75 and 100, are given. The four τ scales, τ_1, τ_2, τ_3 and τ_4, correspond to circular (not rectangular) regions on consecutive frames, in which the numbers of spermatozoa are counted.

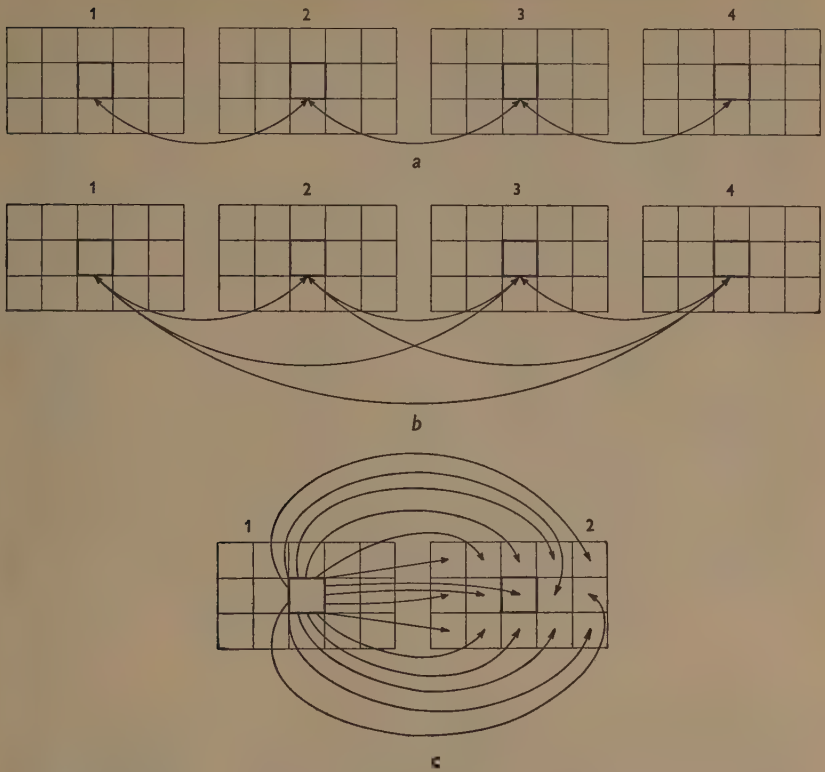

Fig. 4. Three possible ways of comparing the numbers of spermatozoa in selected regions on consecutive frames. A curved line with an arrow on it is a comparison: *a*, the method used in these experiments; *b*, the same as *a*, but region 1 is compared with regions 2, 3, 4, etc., region 2 with regions 3, 4, etc., and so on; *c*, other combinations of comparisons are made between subregions on different frames.

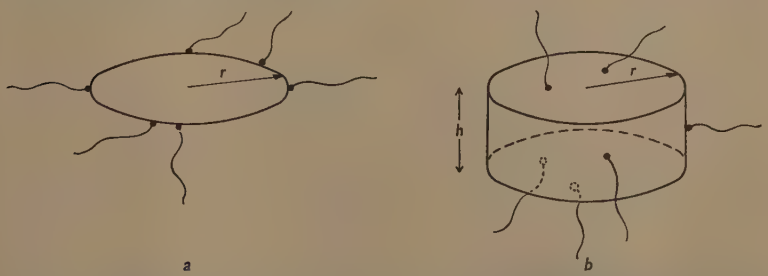

Fig. 5. *a*, spermatozoa 'bombarding' a circle of radius r. The bombardment frequency is $2d\bar{c}r$, where d is the number of spermatozoa per unit area. *b*, spermatozoa 'bombarding' a cylinder of radius r and height h. The bombardment frequency is $\frac{1}{2}D\bar{c}\pi r(r+h)$, where D is the number of spermatozoa per unit volume.

A ciné-film was then taken of the suspension, using a ×18 phase-contrast objective, and a ×10 ocular. The ciné-camera was an Askania 35 mm., with the shutter aperture set at 45°, running at 25·8 frames/sec. The light source was a Point-o-Lite with one Chance heat filter between it and the microscope. In the experiment described in this paper, the suspension was photographed for 20·2 sec., which is equivalent to 521 frames.

All operations were carried out at 37° C. The overall magnification (1590 after projection of the film) was such that the field photographed was a rectangle with sides 337 and 255 μ. When plotting the positions of spermatozoa, or counting them, this rectangular field was subdivided into twenty subrectangles each with sides 67·4 and 63·8 μ.

RESULTS

Random movements of spermatozoa. As mentioned in the section entitled 'Theory', if the spermatozoa move in random directions, their spatial distribution will be random, in which case the numbers in the field will fluctuate Poisson-wise. This will only apply provided the positions of spermatozoa in the fields under examination are uncorrelated, i.e. provided corr (n_1, n_2) is small, say 0·05 or less. The time interval between counts which provides a correlation of 0·05 or less can be calculated for a region of any desired size. In these experiments each frame was divided into twenty subframes or rectangles with sides 67·4 and 63·8 μ. Assuming an average sperm speed of 125 μ/sec.,

$$\text{corr}\,(n_i, n_{i+1}) = e^{-\lambda\tau} = \exp -\left\{\frac{250(67\cdot4+63\cdot8)}{3\cdot1416(67\cdot4)\,(63\cdot8)}\tau\right\} \tag{6}$$

$$= e^{-2\cdot428\tau}. \tag{6·1}$$

If $e^{-\lambda\tau}$ is to equal 0·05, τ must equal 1·23 sec. The film speed being 25·8 frames/sec., every 30th frame will have a sufficiently low correlation to allow the numbers of spermatozoa in each subframe to be examined for a Poisson distribution. If the numbers of spermatozoa in the twenty subframes fluctuate Poisson-wise, the numbers in groups of subframes, for example, in whole frames, will also be Poisson-distributed (Fisher, 1948); but if a whole frame is examined, λ, which *inter alia* is a function of the frame size, and therefore of corr (n_i, n_{i+1}), will have different values from those applicable to the subframe case. To test, therefore, for random movement, we select frames sufficiently far apart in time to examine whole and subframes for a Poisson distribution of sperm numbers. A simple calculation similar to that done above for subframes shows that if whole frames as well as subframes are to be examined, $\tau \doteqdot 4{\cdot}6$ sec. and every 118th frame may be selected. The actual numbers of spermatozoa observed in such an experiment are given in Table 1. The calculations show that the directions of movements of the spermatozoa are random. As mentioned above, it follows automatically that the sperm numbers will fluctuate Poisson-wise in whole as opposed to subframes, and this can easily be verified by the use of Fisher's dispersion index

$$\chi^2_{\nu-1} = \frac{1}{n}\Sigma\,(n_i - \bar{n})^2, \tag{7}$$

or the relationship

$$1+2\sqrt{\left(\frac{2\nu}{(\nu-1)^2}\right)}>\frac{V(n)}{\bar{n}}>1-2\sqrt{\left(\frac{2\nu}{(\nu-1)^2}\right)}, \qquad (8)$$

where ν is the number of observations, in this case 5. The numbers relevant to whole frames are given in Table 1, in the horizontal column which starts with a Σ. Substitution of the appropriate values in these two equations shows that $\chi_4^2=2{\cdot}9044$, making $P=0{\cdot}5$–$0{\cdot}7$, and $2{\cdot}5811>0{\cdot}7261>-0{\cdot}5811$.

Table 1. *Number of spermatozoa in frames and subframes*

Frame no. ...	2	119	237	355	473
Subframe no. 1	5	6	15	9	9
2	5	10	7	2	7
3	5	6	3	5	9
4	4	5	8	8	6
5	8	9	7	5	7
6	7	3	8	9	4
7	7	8	6	6	7
8	6	7	4	7	4
9	6	4	7	4	8
10	6	5	11	6	6
11	11	6	6	9	11
12	6	9	7	7	5
13	3	3	6	5	5
14	5	7	4	0	6
15	4	5	3	3	7
16	3	9	14	13	6
17	3	8	1	4	3
18	10	6	11	6	9
19	13	8	8	8	10
20	3	3	9	13	9
Σ	120	127	145	129	138

Subframe analysis

No. of sperm per subframe	0–3	4	5	6	7	8	9	10–∞
No. of subframes with that number, o	14	9	12	18	14	10	11	12
Expected no. of subframes with that numer, *e*	10·5806	10·7979	14·2316	15·6306	14·7225	12·1284	8·8804	13·0280

$$\chi_6^2=\frac{(o-e)^2}{e}=3{\cdot}1096. \quad P=0{\cdot}7\text{–}0{\cdot}8$$

These observations show that in these conditions, the movements of spermatozoa are essentially random. If the suspension exhibited much orientation, or if a significant number of spermatozoa in the suspension swam in circles, the Poisson-distribution tests would fail and it would be necessary to attempt more recondite methods of analysis.

Distribution of speeds. A new method of estimating the mean speed of a suspension must be compared with some different way of measuring the same

quantity. Accordingly, the 25·8 frames/sec. ciné-film was projected frame by frame on to squared paper and the outline of the head of each spermatozoon was drawn in. The consecutive positions of each head were then joined by straight lines and their lengths measured, from which the speeds of each spermatozoon were calculated. At this film speed and with the magnification used, 1590, the ambiguities about the consecutive positions of particular spermatozoa mentioned earlier did not arise. Mr M. J. Hubbard and Miss G. E. Bending, a research

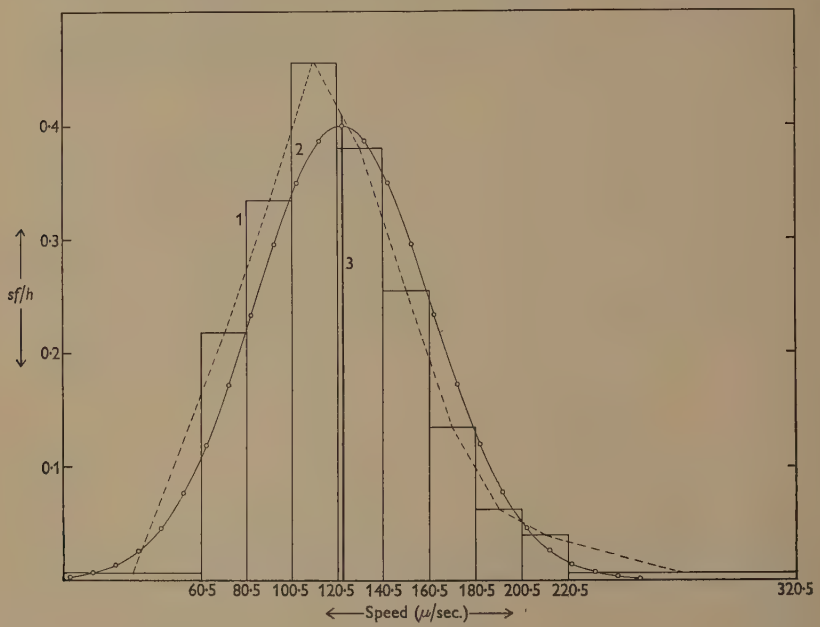

Fig. 6. 1, sperm speed histogram and frequency polygon; 2, normal distribution with the same mean and standard deviation; 3, mean; *s*, standard deviation; *f*, relative frequency; *h*, cell width. Total number of sperm tracks measured, 433.

assistant provided by the Medical Research Council, carried out this tedious and time-consuming work under my direction. The distribution of sperm speeds is shown in Fig. 6. The mean and standard deviation of the distribution are 122·7 and 38·6 μ/sec. The normal distribution with the same mean and standard deviation is included in Fig. 6, to show how much the sperm speed distribution diverges from normality. It will be observed that, as might be expected on theoretical grounds, it is both skew and leptokurtic. There are, of course, a certain number of dead or motionless spermatozoa in the field. If these are taken into consideration, as they are in the probability-after-effect method of estimating the mean speed, the mean becomes 117·2 μ/sec.

Mean speed by probability-after-effect method. To illustrate how calculations are performed, we shall examine the number of spermatozoa in a rectangle with sides $67{\cdot}4\mu$ and $127{\cdot}6\mu$ in the middle of every seventh frame, using frames 2, 9, 16, 23, 30, 37, 44, 51, 58 and 65 for this purpose. This means that k in equ. (5) is 10, which is too low for reasonable precision but convenient for explaining the method. The procedure is shown in Table 2. The estimate of the mean speed

Table 2. *Estimate of mean speed and standard error using probability-after-effect principle*

Frame no.	k	No. of sperm, n, in rectangle	$\lvert\delta\rvert$	δ^2
2	1	9	1	1
9	2	10	5	25
16	3	15	4	16
23	4	11	1	1
30	5	12	0	0
37	6	12	3	9
44	7	9	1	1
51	8	10	4	16
58	9	14	1	1
65	10	15		

$$\overline{\delta^2} = 2\bar{n}(1-e^{-\lambda\tau}) \qquad (2{\cdot}1)$$

$$\lambda = \frac{2\bar{c}(x'+y')}{\pi x'y'} \qquad (3)$$

Film speed, 25·8 frames/sec.

$\tau = 0{\cdot}27$ sec. (every seventh frame)

$x' = 67{\cdot}4$, $y' = 127{\cdot}6$

Substituting,

$$\boxed{\bar{c} = 103{\cdot}7}$$

$$\bar{n} = \frac{1}{k}\Sigma n = 11{\cdot}7 \qquad \overline{\delta^2} = \frac{1}{k-1}\Sigma\delta^2 = 7{\cdot}7778$$

$$V(\bar{c}) = \frac{\bar{c}^2}{k\bar{n}(x \log_e x)^2}\left\{\frac{\bar{n}(1-x)^2(3+x)}{(1+x)} + x(1-x)\right\}, \qquad (5)$$

where $x = e^{-\lambda\tau}$.

Substituting, $V(\bar{c}) = 3872$; standard error 62·2.

Table 3. *Variation in estimate of mean speed, $\bar{c}$, and the standard error, s, of the estimate, with k, the number of sperm counts. $\bar{c}$ and s in μ/sec.*

k	$\bar{c}$	s
10	103·7	62·2
25	76·0	27·2
50	99·0	26·3
75	110·5	24·5

is 103·7 with an associated standard error of 62·2. To get a more precise estimate of $\bar{c}$, the same calculations were carried out, but with $k = 75$. In this case $\bar{n} = 12{\cdot}0133$ and $\overline{\delta^2} = 8{\cdot}3649$, which, on substitution in equ. (2·1), provide an estimate of $\bar{c}$ equal to $110{\cdot}5\,\mu$/sec., which is in good agreement with the value obtained by the 'plotting method'. The standard error was 24·5. It is of interest to examine how in practice $\bar{c}$ and its precision vary with k; this is done in Table 3. In the first case examined, multiplication of the standard error by 1·6449, obtained from the normal tables, determines an interval within which $\bar{c}$ lies with a fiducial probability of 90%; but one end of this interval is negative. Negative values of $\bar{c}$ have no meaning

and arise because, for small k, the distribution of the estimate of $\bar{c}$ departs from normality.

Homogeneity. If the probability-after-effect method of measuring the mean speed of a suspension is used in a series of experiments, it is of course important to establish that the sampling procedure is satisfactory. 'Sampling procedure' refers to everything that happens between collecting the original ejaculate and estimating the mean speed of the test suspension. Suppose, for example, that we estimate the mean speeds of spermatozoa in five subsamples, derived by 'identical'

Table 4. *Estimate of mean speed, $\bar{c}$, and variance, $V(\bar{c})$, in five subsamples taken consecutively from one sample of semen diluted 1/4 with fructose phosphate buffer*

$\bar{c}$	$V(\bar{c})$
133·46	473·69
145·35	701·68
136·49	589·98
112·51	371·26
122·43	392·77

methods from a parent sample (Table 4). If the sampling procedure is satisfactory, these five mean speeds should be mutually consistent and this can be subjected to quantitative verification. The best joint or pooled estimate of $\bar{c}$ from the data in Table 4 is

$$\bar{c}' = \sum_{i=1}^{5} I_i \bar{c}_i \Big/ \sum_{i=1}^{5} I_i, \tag{9}$$

where $I_i = 1/V(\bar{c}_i)$. $\sum_{i=1}^{5} I_i(\bar{c}_i - \bar{c}')^2$ is distributed as χ^2 on four degrees of freedom, and substitution of the values in Table 4 shows that $\chi_4^2 = 1{\cdot}34$ from which P lies between 0·80 and 0·90. This means that there are inadequate grounds for rejecting the hypothesis that the five estimates of mean speed were obtained from one parent sample; in other words, the subsampling procedure was satisfactory.

PRACTICAL CONSIDERATIONS

As this method of estimating the mean speed, i.e. the *true* activity, of a sperm suspension may be of some practical interest, the details of making measurements and estimating mean speeds from them are summarized in this section.

(1) Take a ciné-film of the sperm suspension, preferably but not necessarily diluted 1/4. The film speed should be between 5 and 10 frames/sec. Phase-contrast lenses should be used. A cover-slip should be gently placed on top of the drop of sperm suspension, as described in the section 'Experimental procedure'. Alternatively, fragments of any innocuous material of suitable thickness can be placed between the cover-slip and the microscope slide to keep the depth of the suspension constant.

(2) Select a region of known area and perimeter in the microscope field (on the ciné-film).

(3) Find the value of t which makes λt equal to or greater than 2·996. In the case of bull semen, if the region (see (2)) is a rectangle with sides x' and y',

$\lambda = 64(x'+y')/x'y'$. For a square with side x', $\lambda = 128/x'$; and for a circle of radius r, $\lambda = 64/r$.

(4) Divide up the field into about twenty subregions of equal area and perimeter and test whether the numbers of spermatozoa in these subregions are distributed Poisson-wise (as in Table 1, p. 187), using frames which are t sec. apart, t having been determined in (3). Assuming that the Poisson distribution test is positive, proceed as follows.

(5) Select a region whose area is such that on the average it contains about ten spermatozoa. The size of the region depends on the microscope magnification, the sperm density and the method of projecting the film. If the semen contains about 10^9 sperm/ml. and is diluted 1/4, a rectangle with sides 70 and 125 μ or a circle with radius 50 μ will contain about ten spermatozoa.

(6) Count the number of spermatozoa in this region on k consecutive frames. If $k=75$ and the average number of spermatozoa in the region is about ten, this will take some 2 hr.

(7) Calculate $\bar{n}$, the average number of spermatozoa in the region, and $\overline{\delta^2}$, the average of the square of the differences between the number of spermatozoa in regions on consecutive frames.

(8) If the region is a rectangle with sides x' and y', a square with side x', or a circle of radius r, the mean speed of the sperm suspension, $\bar{c}$, is given by

$$\left.\begin{aligned} \bar{c} &= -\frac{\pi x'y'}{2\tau(x'+y')}\log_e\left(1-\frac{\overline{\delta^2}}{2\bar{n}}\right), \\ \bar{c} &= -\frac{\pi x'}{4\tau}\log_e\left(1-\frac{\overline{\delta^2}}{2\bar{n}}\right), \\ \bar{c} &= -\frac{\pi r}{2\tau}\log_e\left(1-\frac{\overline{\delta^2}}{2\bar{n}}\right), \end{aligned}\right\} \qquad (10)$$

where τ is the time interval between successive photographs.

(9) The precision or accuracy of the estimate of $\bar{c}$ depends on the average number $\bar{n}$ of spermatozoa in the region of the field selected for examination, the time interval τ between successive photographs, the number of photographs examined k, and the size of the region. These facts are illustrated in Fig. 7, which requires some explanation. Consider the curve labelled IV. Any point on this curve has associated with it a particular value of $\bar{n}$ and of τ_1. For example, the point P has co-ordinates $\bar{n}=2$ (the antilogarithm of 0·3), and $\tau_1 = 0{\cdot}114$ sec. All pairs of values of $\bar{n}$ and τ_1 on this curve will provide estimates of $\bar{c}$ with the same variance 8000 (if ten photographs are examined), or standard error 89·4. A user of the curves in Fig. 7 decides on the variance which he considers adequate. The relevant curve provides a set of alternative values of $\bar{n}$ and τ_1 which will give estimates of $\bar{c}$ with the desired variance. The variance decreases as the number of frames counted is increased. Each curve in Fig. 7 is therefore labelled according to the variance associated with it *and* according to k, the number of frames counted. Four different values of k, 10, 25, 75 and 100, are considered.

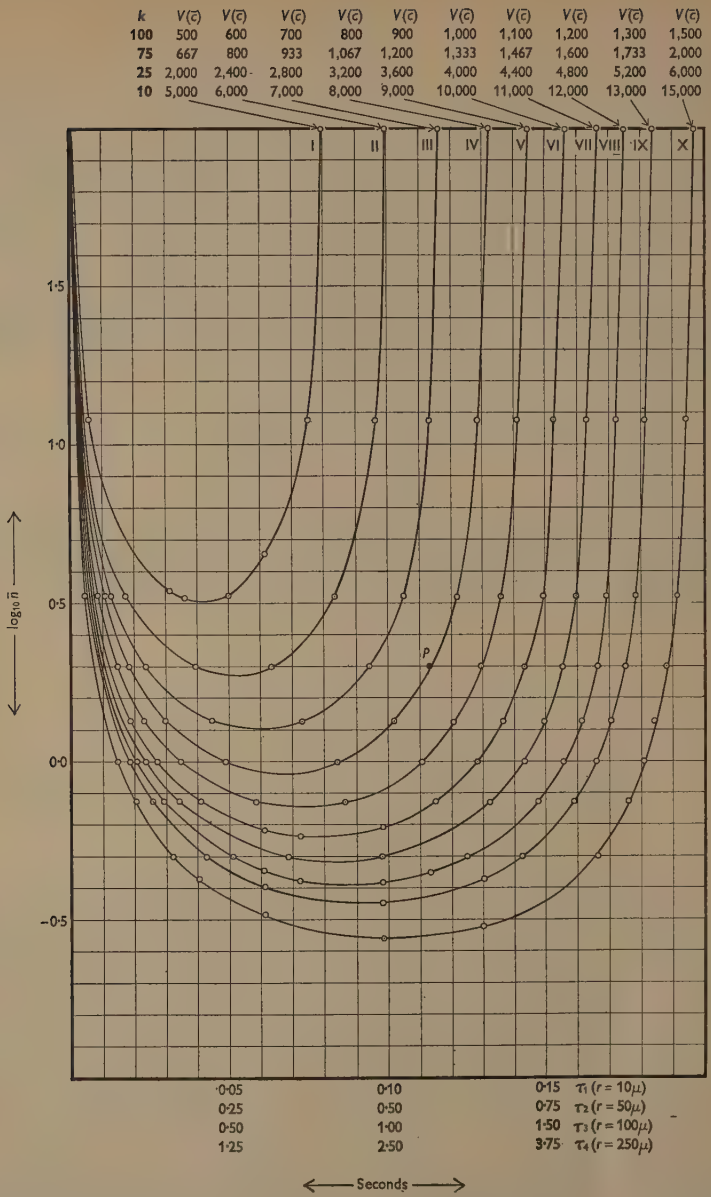

Fig. 7. The relationships between $\bar{n}$, the average number of spermatozoa counted in a circular region of known radius r, and the time interval τ between consecutive photographs. Any point on any one of the curves I–X provides values of $\bar{n}$ and τ, which, when substituted in the equation for the estimate of mean speed, will produce an estimate of $\bar{c}$ with known variance $V(\bar{c})$. The variances appropriate to curves I–X are given above the curves, in four rows, according to k, the number of counts of spermatozoa made. The four τ scales correspond to circular regions of different radii.

We must now consider how the variance changes with $\bar{n}$ and τ. For the purposes of tabulating variances as in Fig. 7, it is convenient to consider circular regions. The four abscissa scales, τ_1, τ_2, τ_3 and τ_4, correspond respectively to circular regions with radii 10, 50, 100 and 250 μ.

The curves in Fig. 7 are based on a mean speed of 110·5 μ/sec. In practice, however, Fig. 7 should be used as a guide, which will save much time, to the conditions of the experiment. The actual variance relevant to an experiment should be estimated by means of the equation

$$V(\bar{c}) = \frac{\bar{c}^2}{k\bar{n}(x \log_e x)^2}\left\{\frac{\bar{n}(1-x)^2(3+x)}{(1+x)} + x(1-x)\right\},$$

where $x = e^{-\lambda\tau}$. There are, of course, many published tables giving the values of e^{-z} for different values of z, from which $e^{-\lambda\tau}$ can be quickly calculated.

(10) A simple method of testing the adequacy or inadequacy of the sampling procedure is given in the section entitled 'Homogeneity' on p. 190.

DISADVANTAGES OF THE METHOD

The main disadvantage is the expense involved in taking ciné-micrographs as opposed to photo-micrographs. It is doubtful whether the size of the region examined can be increased sufficiently, by reduction of magnification, to make τ suitable for ordinary photo-micrography. Figs. 7 and 3 are deceptive in this respect, as they superficially suggest that 5 sec. intervals between photographs might be achieved. This is not the case unless the sperm suspension is over-diluted. Whether the refinements outlined in the paragraph headed 'Loss of Information' on p. 183 will make it possible to have 5–10 sec. intervals between photographs, thus obviating the need for cinematography, is not yet known.*

APPENDIX

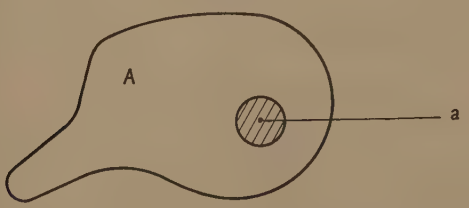

Fig. 8.

1. *Consequences of the assumption of random movement*

Let there be n spermatozoa in A (Fig. 8). If the movements of the spermatozoa are random, the probability that a particular spermatozoon will, at some particular instant, be in a, is equal to a/A. This statement implies random location of spermatozoa which is a consequence of their random movement.

* In any case, the longer the time interval between photographs, the greater is the chance of the 'mean free time' of the spermatozoa being shorter than the time interval, with consequent underestimation of the mean speed.

The probability $\Pr\{x\}$ that the number of spermatozoa observed in a at some instant of time will be x is given by

$$\Pr\{x\} = \binom{n}{x}\left(\frac{a}{A}\right)^x\left(1-\frac{a}{A}\right)^{n-x} \tag{1}$$

$$= \frac{n!}{x!\,(n-x)!}\left(\frac{a}{A}\right)^x\left(1-\frac{a}{A}\right)^{n-x} \tag{1·1}$$

$$= \frac{1}{x!}n(n-1)(n-2)\ldots(n-\overline{x-1})\left(\frac{a}{A}\right)^x\left(1-\frac{a}{A}\right)^{n-x} \tag{1·2}$$

$$= \frac{1}{x!}\frac{n(n-1)(n-2)\ldots(n-\overline{x-1})}{n^x}\left(n\frac{a}{A}\right)^x\left(1-\frac{a}{A}\right)^{n-x} \tag{1·3}$$

$$= \frac{1}{x!}1\left(1-\frac{1}{n}\right)\left(1-\frac{2}{n}\right)\ldots\left(1-\frac{x-1}{n}\right)\left(n\frac{a}{A}\right)^x\left(1-\frac{a}{A}\right)^{n-x}. \tag{1·4}$$

Let $A\to\infty$ and $n\to\infty$ in such a way that the average number of spermatozoa per unit area remains finite, i.e. $\lim\limits_{\substack{n\to\infty\\A\to\infty}}\frac{n}{A}=k\ (k\neq 0)$. Then

$$\Pr\{x\}\to\frac{1}{x!}1(ka)^x\left(1-\frac{ka}{n}\right)^n\left(1-\frac{ka}{n}\right)^{-x} \tag{2}$$

$$\to\frac{1}{x!}(ka)^x e^{-ka}\times 1 \tag{2·1}$$

$$\to\frac{1}{x!}\lambda^x e^{-\lambda},\quad \lambda=ka. \tag{2·2}$$

x is therefore distributed Poisson-wise with parameter ka, which equals the average number of spermatozoa in a.

2. *Proof that* $E(\delta^2)=2E(n)\,(1-e^{-\lambda\tau})$

(1) Let the number of spermatozoa observed in an area a be n_1 at time t_1, and n_2 at time t_2, where $t_2-t_1=\tau$.

(2) Let $n_1-n_2=\delta$.

(3) Let the probability that a particular spermatozoon, in a at time t, will leave in the interval $(t,\ t+\delta t)$ be $\lambda\delta t+o(\delta t)$.

(4) Let the probability that one spermatozoon will arrive in a during $(t,\ t+\delta t)$ be $\mu\delta t+o(\delta t)$.

Note. (3) and (4) imply that the spermatozoa do not drift or age.

(5) Let $E(n)$ be the average number of spermatozoa in a.

The average number of spermatozoa leaving a in δt is $\lambda E(n)\,\delta t$, while the average number entering a in the same time is $\mu\,\delta t$. When the system is in equilibrium,

$$\lambda E(n)=\mu. \tag{1}$$

Since $E(n_1)=E(n_2), \quad V(n_1)=E(n_1^2)-E^2(n_1), \quad V(n_1)=V(n_2)$

and $$\text{corr}\,(n_1, n_2)=\{E(n_1 n_2)-E(n_1)\,E(n_2)\}/\{V(n_1)\,V(n_2)\}^{\frac{1}{2}},$$

$$E(\delta^2)=2V(n_1)\{1-\text{corr}\,(n_1, n_2)\}. \tag{2}$$

If the number of spermatozoa in a is distributed Poisson-wise, $V(n_1)=E(n_1)=E(n)$ and $\text{corr}\,(n_1, n_2)=e^{-k\tau}$, where k is a constant.* To determine k, consider an infinitesimal value of τ, δt, so that $e^{-k\tau}=1-k\delta t+o(\delta t)$. Let n_1 and n_2 be the number of spermatozoa present at t and $t+\delta t$ and $p(n_1, n_2)$ be the probability of these numbers. δt is selected in such a way (i.e. so small) that the only values of $p(n_1, n_2)$ which are not zero are

$$\left.\begin{aligned} p(n_1, n_1+1)&=p(n_1)\,\mu\,\delta t,\\ p(n_1, n_1-1)&=p(n_1)\,\lambda n_1\,\delta t,\\ p(n_1, n_1)&=p(n_1)\{1-(\mu+\lambda n_1)\,\delta t\}. \end{aligned}\right\} \tag{3}$$

Note. For fixed n_1, the average number of spermatozoa leaving a in δt is $\lambda n_1 \delta t$, while the average number entering a is $\mu\,\delta t$. Hence the probability of none entering and none leaving a in δt is $1-(\mu+\lambda n_1)\,\delta t$.

Using equs. (1) and (3),

$$E(n_1 n_2)=-\lambda E(n)\,\delta t+E(n)+E^2(n). \tag{4}$$

As in a Poisson distribution

$$\text{corr}\,(n_1, n_2)=\{E(n_1 n_2)-E^2(n)\}/E(n). \tag{5}$$

From equ. (4) $$\text{corr}\,(n_1, n_2)=1-\lambda\delta t. \tag{6}$$

But $$\text{corr}\,(n_1, n_2)=e^{-k\tau}=1-k\,\delta t.$$

Hence $k=\lambda$ and $$E(\delta^2)=2E(n)\,(1-e^{-\lambda\tau}). \tag{7}$$

Consider a spermatozoon initially within a. Divide the time interval $t, t+\tau$ into N subintervals of length τ/N. The probability $(1-P)$ that the spermatozoon will remain within a, *throughout the interval under consideration*, is

$$\lim_{n\to\infty}\left(1-\frac{\lambda\tau}{N}\right)^N=e^{-\lambda\tau}. \tag{8}$$

Hence $$E(\delta^2)=2E(n)\,P, \tag{9}$$

where P is the probability that a spermatozoon will have left a during the interval, but may be back again in a at the end of the interval. Chandrasekhar defines P in a different and slightly less accurate way. Mr D. V. Lindley, of the Statistical Laboratory, Cambridge, drew my attention to this point.

3. *Meaning of* λ

Let $Y=$ number of spermatozoa arriving in a in δt, Y taking values 0, 1, 2, ...

If δt is sufficiently small, $$\Pr\{Y=1\}=\mu\,\delta t, \tag{1}$$

$$\Pr\{Y=0\}=1-\mu\,\delta t, \tag{2}$$

* See Appendix, 4, for the proof of this relationship.

$\Pr\{Y=m\}$, $m \geqslant 2$ being neglected,

$$E(Y)=(0\times\overline{1-\mu\delta t})+(1\times\mu\delta t), \tag{3}$$

$$=\mu\delta t. \tag{3·1}$$

$E(Y)$ is the average number of spermatozoa arriving in a in δt. But from kinetic theory, the average number of spermatozoa which collide with the periphery of a, i.e. arrive in a, in δt is

$$Z\delta t l, \tag{4}$$

where $Z=d\bar{c}/\pi$ and l is the length of the periphery of a. d is the number of spermatozoa per unit area.

Hence $$\mu\delta t=Z\delta t l, \tag{5}$$

or $$\mu=Zl.* \tag{5·1}$$

Suppose a is a rectangle with sides x' and y'. Then

$$l=2(x'+y') \quad \text{and} \quad \mu=2d\bar{c}(x'+y')/\pi.$$

Now $\lambda=\mu/\nu$, where $\nu=dx'y'$. Then

$$\lambda=\frac{2\bar{c}(x'+y')}{\pi x'y'}, \tag{6}$$

or, in the case of a square, $$\lambda=\frac{4\bar{c}}{\pi x'}. \tag{6·1}$$

If a is a circle of radius r, $$\lambda=\frac{2\bar{c}}{\pi r}. \tag{7}$$

4. *Collision frequency in two dimensions*

Consider a cm.^{2} of a suspension of moving spermatozoa. Each spermatozoon has a velocity vector associated with it and each spermatozoon will have a representative point in velocity space with rectangular coordinates c_x, c_y. An element of area, surrounding a typical point $\mathbf{c}$ in velocity space, is denoted by $\delta\mathbf{c}$. The points in velocity space will have a particular density, $f(\mathbf{c})$, which in general will vary from point to point and time to time. The number of points in $\delta\mathbf{c}$ will be $f(\mathbf{c})\,\delta\mathbf{c}$. If instead of a unit area we start with an element of area $\delta\mathbf{r}$, the number of points in $\delta\mathbf{c}$ will be $f(\mathbf{c})\,\delta\mathbf{c}\,\delta\mathbf{r}$. In $\delta\mathbf{c}$ there will be $f(\mathbf{c})\,\delta\mathbf{c}\,\delta\mathbf{r}$ spermatozoa with velocities in the velocity class $\delta\mathbf{c}$. The relationship between the velocity space density and the spermatozoa's speed frequency function $g(c)$ is, for unit area and an annulus $\delta\mathbf{c}$ of width δc,

$$f(\mathbf{c})\,\delta\mathbf{c}=dg(c)\,\delta c, \tag{1}$$

where d = number of spermatozoa per unit area. Equ. (1) can be written

$$f(\mathbf{c})=\frac{dg(c)}{2\pi c}. \tag{1·1}$$

* There are certain mathematical assumptions 'hidden' in this equation; they will be discussed elsewhere, as will be the implications of the approximate nature of the model.

Consider an infinitesimal element of length ds, with spermatozoa moving through ds, with velocities in the velocity class $\delta\mathbf{c}$. The number of spermatozoa of class $\delta\mathbf{c}$ in a strip of area $ds\cos\psi c\,dt$ will be

$$f(\mathbf{c})\,dsc\cos\psi\,dt\,d\mathbf{c} \tag{2}$$

$$=f(\mathbf{c})\,dsc_n\,dt\,d\mathbf{c}, \tag{2·1}$$

where c_n = component of c in the direction perpendicular to ds and $\delta\mathbf{r}=ds\cos\psi c\,dt$. The number of spermatozoa N of all classes passing from one side of ds to the other, in the same direction (i.e. $c_n>0$), is

$$N=ds\,dt\int_{c_n>1}f(\mathbf{c})\,c_n\,d\mathbf{c}. \tag{3}$$

Making c_n identical with c_y,

$$N=ds\,dt\int_{c_y>0}f(\mathbf{c})\,c_y\,d\mathbf{c} \tag{3·1}$$

$$=ds\,dt\int_{c_y>0}\frac{dg(c)}{2\pi c}c_y\,d\mathbf{c}. \tag{3·2}$$

In polar co-ordinates

$$N=\frac{d\,ds\,dt}{2\pi}\int_0^\infty\int_0^\pi g(c)\,c\sin\theta\,d\theta\,dc. \tag{3·3}$$

The number of spermatozoa passing through unit length in unit time (from one side to the other) is therefore

$$Z=\frac{d}{\pi}\int_0^\infty cg(c)\,dc \tag{4}$$

$$=\frac{d\bar{c}}{\pi}. \tag{4·1}$$

5. corr $(n_1, n_2)=e^{-kt}$

Let n_1, n_2, n_3 spermatozoa be observed at t, $t+t_1$, $t+t_1+t_2$. Let $p(n_1 \mid n_2)$ denote the probability of n_1 at t, given n_2 at $t+t_1$ (and other expressions similarly, e.g. $p(n_3 \mid n_2, n_1)$). Then

$$p(n_3 \mid n_2)=p(n_3 \mid n_2, n_1) \tag{1}$$

and $E(n_1n_3 \mid n_2)$, the average of n_1n_3 for fixed n_2,

$$=\sum_{n_1=0}^{\infty}\sum_{n_3=0}^{\infty}n_1n_3\,p(n_1, n_3 \mid n_2) \tag{2}$$

$$=E(n_1 \mid n_2)\,E(n_3 \mid n_2). \tag{3}$$

Hence the partial correlation coefficient of n_1 and n_3 for fixed n_2 is zero and by standard formulae for partial correlation coefficients,

$$\text{corr}\,(n_1, n_3)=\text{corr}\,(n_1, n_2)\,\text{corr}\,(n_2, n_3), \tag{4}$$

provided $E(n_2 \mid n_1)=an_1+b$.

The implication of equ. (1) is that once n_2 *is known*, n_1 has no predictive value in relation to n_3. This is known as the Markov property (see, for example, Bartlett, 1950).

Let $g(t)$ be the correlation coefficient of a system of spermatozoa at two times, t apart. Equ. (4) can be written

$$g(t_1+t_2)=g(t_1)\,g(t_2), \quad \text{for all } t_1 \text{ and } t_2 \geqslant 0. \tag{5}$$

The only solution of this equation with $g(0)=1$ and $g(t)\leqslant 1$ is

$$g(t)=e^{-kt}. \tag{6}$$

SUMMARY

1. A new and quantitative method of estimating the average speed of a suspension of spermatozoa has been developed. The method, in which the phenomenon known as probability-after-effect and kinetic theory principles are made use of, can be applied to any system of organisms which move in random directions, in one, two or three dimensions.

2. The experimental procedure is to place a drop of sperm suspension on a microscope slide with a cover-slip on top of the drop, and photograph the spermatozoa at known time intervals.

3. The number of spermatozoa in a region of known size is counted on each photograph. The estimated mean or average speed of the sperm suspension, $\bar{c}$, is given by the equation

$$\bar{c}=-(\pi r/2\tau)\log_e\left\{1-\frac{\overline{\delta^2}}{2\bar{n}}\right\},$$

where r is the radius of a circular region in which the number of spermatozoa is counted, τ is the time interval between photographs, $\overline{\delta^2}$ is the average of the square of the differences between the numbers of spermatozoa counted on consecutive photographs, and $\bar{n}$ is the average number of spermatozoa in the circular region.

4. Appropriate formulae for non-circular regions and for the precision of estimates of $\bar{c}$ are given.

5. A method of testing whether the directions of movement of spermatozoa are random has been applied to bull semen diluted 1/4 with phosphate buffer containing fructose. The movements were found to be random.

6. The distribution of sperm speeds was determined and found to be somewhat skew and leptokurtic, with mean 123 μ/sec. and standard deviation 39. If dead or motionless spermatozoa were included, the mean speed became 117 μ/sec.

7. Using the probability-after-effect equation given in (3), the mean speed of the suspension, including dead or motionless spermatozoa, was found to be 111 μ/sec., with a standard error of 24·5. The standard error and therefore the precision of the estimate is under the control of the experimenter.

8. Practical instructions for carrying out measurements are summarized in a separate section.

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THE EFFECT OF WASHING ON THE MOTILITY AND METABOLISM OF RAM, BULL AND RABBIT SPERMATOZOA

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The technique of washing tissues and cells has been widely practised in the field of enzyme chemistry, for removing natural substrates and for demonstrating important components of enzyme systems.

In the field of reproductive physiology several authors (e.g. Lardy & Phillips, 1943; Mann, 1945) have used spermatozoa washed in calcium-free Ringer's solution for studying the endogenous metabolism of the cells and to observe the effect of added substances. A further application of the technique in this field is due to Emmens & Swyer (1948) who have used repeated washing in Baker's solution to remove seminal plasma constituents and so presumably to simulate the effect of dilution on rabbit spermatozoa. The method of washing spermatozoa in all cases has consisted of centrifuging either the neat or diluted semen, withdrawing the supernatant and replacing it by a suitable diluent.

In this investigation, the effect of one and of two washings on the motility, oxygen uptake and aerobic glycolysis of ram, bull and rabbit spermatozoa has been studied in a sodium phosphate-fructose diluent at 37° C. The object of the experiment was twofold: first, to gain information on the extent to which mammalian spermatozoa are likely to be affected by procedures normally adopted in the preparation of cell suspensions for metabolic studies, and secondly, assuming provisionally that washing and dilution produce similar effects, to obtain evidence as to a metabolic basis for the adverse effect of dilution on mammalian spermatozoa (Salisbury, Beck, Cupps & Elliot, 1943; Emmens & Swyer, 1948; Cheng, Casida & Barrett, 1949). This should not be confused with the dilution effect described by Gray (1928), who found that the motility and respiratory rate of sea-urchin spermatozoa was increased by dilution with sea water.

MATERIALS AND METHODS

Bull and rabbit semen was collected by means of the artificial vagina, and ram semen by electrical stimulation as described by Gunn (1936). In all cases only apparently normal specimens with good motility were employed. Experiments with ram and rabbit semen were started immediately after collection and those with bull semen within 2 hr. of collection. The semen was stored during this

period at about 10° C., the usual slow cooling precautions being taken to avoid cold shock.

The procedure each day was to dilute the semen (which in most cases was composed of pooled ejaculates) approximately 1 in 3 with diluent to give a volume of about 10 ml. in a graduated centrifuge tube. Duplicate 1 and 0·5 ml. aliquots of the unwashed sperm suspension were then further diluted 1 in 3 with the same diluent in Warburg flasks and small tubes respectively—the flasks being used for the measurement of oxygen uptake, and the tubes, which were shaken in the Warburg bath with the flasks, for estimations of lactic acid production and observations of motility. The remaining, unwashed sperm suspension in the centrifuge tube was then spun at 1500 r.p.m. (about 300 g.) for 10 min., the supernatant withdrawn and replaced by an equal volume of diluent. Aliquots of the once-washed sperm suspension were taken as before and the washing procedure repeated. Early experiments indicated the importance of thoroughly dispersing the spermatozoa in the diluent after centrifuging. A satisfactory method of doing this without damaging the cells was found to be by sucking the sperm up and down in a wide-bore Pasteur pipette fitted with a rubber teat. An isotonic diluent of pH 7·0 containing 0·032M-$NaH_2PO_4.H_2O$, 0·048M-$Na_2HPO_4.12H_2O$, 0·040M-NaCl and 0·022M-fructose was found to be adequate to maintain a fairly constant pH within 0·1 unit of the initial value. The diluent was freshly prepared each day by the appropriate dilution of 0·4M stock solutions of the A.R. salts, with glass-distilled water, solid fructose being added to give a concentration of 0·4% (w/v). Stock phosphate and chloride solutions were stored in a refrigerator and discarded at the first signs of mould growth. The pH of all diluents was checked with the glass electrode prior to use.

Each experiment was of 5 hr. duration, with the Warburg bath operating at 37° C. and a shaking rate of 114 strokes per minute, the amplitude of the stroke being 4 cm. Oxygen uptake and motility observations were made at hourly intervals and the lactic acid production calculated over the 5 hr. period from estimations made at the start and end of experiments. In the washing experiments all observations and analyses were made in duplicate on each ejaculate, the mean values being recorded in the tables of results.

Oxygen uptake was measured by the direct Warburg technique described by Umbreit, Burris & Stauffer (1949) using 0·2 ml. of 20% (w/v) KOH for the absorption of CO_2. Flasks of 22–35 ml. volume were used in some early experiments, but these were subsequently replaced by smaller ones of 12–16 ml. capacity in order to increase the sensitivity of the readings. Both types were calibrated in duplicate using mercury (Dixon, 1943). Agreement between duplicate calibrations was good, the mean between-duplicate difference for thirty-five flasks being $0{\cdot}14 \pm 0{\cdot}15\%$ of the mean volume, indicating that the contribution of calibration to the overall error of the Warburg technique is negligible. The calibration of the flasks and the accuracy of the manometric technique was further checked by setting up flasks containing a mixture of 1 ml. of 5% (v/v) H_2SO_4 and 1 ml. of 0·1 vol. H_2O_2 in the body of the flask with 0·1N-$KMnO_4$ in a side-arm. After equilibration the

permanganate was tipped into the acidified peroxide and the gas production recorded after 1 hr. Experiments on two different days, involving twelve and ten flasks respectively, gave readings of $196{\cdot}2 \pm 5{\cdot}1$ and $191{\cdot}8 \pm 5{\cdot}3$ μl. There was no evidence of bias with respect to any one manometer. Standard deviations of a similar order were obtained in preliminary experiments in which the oxygen uptake of ram spermatozoa was measured. Thus in two experiments, each of 1 hr. duration and each involving ten flasks, the mean oxygen uptakes were $49{\cdot}0 \pm 4{\cdot}5$ and $77{\cdot}0 \pm 4{\cdot}5$ μl. respectively. The corresponding standard errors are 1·4 and 1·5 μl., which compare favourably with a mean standard error of 3·9 μl. quoted by Comstock, Green, Winters & Nordskog (1943) for four trials with ram spermatozoa, each involving six flasks.

Lactic acid was estimated by the method of Barker & Summerson (1941). Duplicate analyses, on the same lactic acid sample, in most cases did not differ by more than 0·20 μg., which is similar to the accuracy stated by Umbreit *et al.* (1949). Three tests, each involving duplicate estimations on at least eight different concentrations of standard lithium lactate, indicated that the colour development obeyed the Beer-Lambert law over a final lactate concentration of 0–8 μg. The stability of the colour was found to be good, duplicate tests made on nine equispaced different lactate concentrations over the above range showing no significant variation in transmission during a 3 hr. period. A check on the spectral-transmission graph in which duplicate readings were made on three different days on 2 μg. lactate standards showed that the wave-length of maximum light absorption was 570 mμ. The wave-length of maximum absorption according to the originators of the method is 565 mμ. As a routine procedure six estimations on four different concentrations of standard lithium lactate were made with each batch of unknowns and a new calibration curve constructed on each occasion. An analysis of variance, using the within-duplicate mean square as the test variance, showed that there was no significant variation either between slopes or between positions of the curves.

Motility observations were made according to the system of Emmens (1947), full motility being scored as 4 and complete immotility as zero.

Spermatozoa counts, using an improved Neubauer haemocytometer under high power, were made on duplicate samples taken at the appropriate stage during the washing procedure and diluted with 3% (w/v) sodium chloride. The difference between duplicate counts on sixty-four semen samples exceeded twice the theoretical standard error on twelve occasions; thus any departure from expectation in the spermatozoa counting was not serious.

Samples were taken from the Warburg flasks at the end of some experiments for bacterial counts. These samples were sealed in phials and stored in solid carbon dioxide until the actual counts were made. The technique adopted for counting bacteria was a direct one, similar to that for spermatozoa, except that phase-contrast illumination was used.

RESULTS

The effect of washing once was studied on four pooled ejaculates from each of the three species, and the effect of washing twice on eight pooled ejaculates from rams and four each from bulls and rabbits.

In the analyses of variance for the oxygen uptake, lactic acid production and motility data, the figure for the total 5 hr. period has been used as the unit observation.

The results of the experiments with the once- and twice-washed ram spermatozoa have been analysed separately because of unequal numbers of replicates, whilst

Table 1. *Hourly motility score of ram spermatozoa incubated in phosphate-fructose diluent at* 37° C. *and* pH 7·0 *for* 5 *hr.*

Treatment	Ejaculate	Zero time	1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	Total
Unwashed	1	3·75	3·75	3·50	2·00	0	0	13·00
	2	4·00	4·00	4·00	3·75	1·75	0·50	18·00
	3	4·00	4·00	4·00	4·00	4·00	4·00	24·00
	4	4·00	4·00	4·00	3·75	3·00	1·50	20·25
	5	3·75	3·50	3·00	3·25	3·00	2·75	19·25
	6	4·00	4·00	4·00	4·00	4·00	4·00	24·00
	7	4·00	4·00	4·00	4·00	4·00	4·00	24·00
	8	4·00	4·00	3·00	2·75	2·75	2·25	18·75
Mean		4·00	4·00	3·75	3·50	2·75	2·50	20·50
Once-washed	1	3·75	3·50	2·50	2·00	0·75	0	12·50
	2	4·00	4·00	4·00	3·75	2·00	0·50	18·25
	3	4·00	3·75	3·75	3·75	3·25	2·50	21·00
	4	4·00	4·00	4·00	3·50	3·50	3·00	22·00
Mean		4·00	3·75	3·50	3·25	2·50	1·50	18·50
Twice-washed	1	3·00	2·50	1·25	0	0	0	6·75
	2	3·75	3·75	1·50	0·75	0	0	9·75
	3	3·75	3·50	2·75	2·00	0·75	0·25	13·00
	4	4·00	4·00	3·75	3·25	2·25	2·00	19·25
	5	3·50	3·50	2·25	1·50	1·00	0·50	12·25
	6	4·00	4·00	4·00	4·00	4·00	4·00	24·00
	7	4·00	4·00	4·00	4·00	4·00	4·00	24·00
	8	4·00	4·00	2·75	2·75	1·00	0·25	14·75
Mean		3·75	3·75	2·75	2·25	1·75	1·25	15·50

with the bull and rabbit data, where both groups have equal numbers of replicates, they have been analysed together. In each case the interaction mean square has been used as the error term.

Motility

Tables 1, 2 and 3 give the hourly motility scores for ram, bull and rabbit spermatozoa respectively and Table 4 the summary of the analyses of variance.

Ram. Washing once had little effect, but washing twice depressed motility ($P < 0{\cdot}05$). Ejaculates varied significantly in performance.

Bull. No significant effect was seen on washing, nor did significant differences occur between ejaculates.

Rabbit. Again washing had no significant effect, but highly significant variation occurred between ejaculates.

Table 2. *Hourly motility score of bull spermatozoa incubated in phosphate-fructose diluent at* 37° C. *and* pH 7·0 *for* 5 *hr.*

Treatment	Ejaculate	Zero time	1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	Total
Unwashed	1	4·00	4·00	3·75	3·75	3·00	2·75	21·25
	2	4·00	4·00	4·00	4·00	3·75	3·25	23·00
	3	4·00	4·00	4·00	2·50	1·50	0·75	16·75
	4	4·00	4·00	3·25	2·75	1·25	0·50	15·75
Mean		4·00	4·00	3·75	3·25	2·50	1·75	19·25
Once-washed	1	4·00	4·00	3·75	3·75	3·50	1·75	20·75
	2	4·00	4·00	4·00	4·00	3·75	3·25	23·00
	3	4·00	4·00	4·00	3·00	1·00	0·50	16·50
	4	4·00	4·00	4·00	4·00	3·50	3·00	22·50
Mean		4·00	4·00	4·00	3·75	3·00	2·25	20·00
Twice-washed	1	4·00	4·00	3·25	3·00	1·00	0·25	15·50
	2	4·00	4·00	4·00	4·00	3·75	2·50	22·25
	3	4·00	4·00	4·00	4·00	2·00	1·00	19·00
	4	4·00	3·25	2·50	1·50	0·50	0·50	12·25
Mean		4·00	3·75	3·50	3·25	1·75	1·00	17·25

Table 3. *Hourly motility score of rabbit spermatozoa incubated in phosphate-fructose diluent at* 37° C. *and* pH 7·0 *for* 5 *hr.*

Treatment	Ejaculate	Zero time	1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	Total
Unwashed	1	4·00	4·00	4·00	3·75	2·50	2·50	20·25
	2	4·00	3·25	3·25	2·50	2·50	2·25	17·75
	3	3·25	2·50	2·50	2·50	2·25	1·50	14·50
	4	3·50	3·50	3·25	2·50	2·50	2·25	17·50
Mean		3·75	3·25	3·25	2·75	2·50	2·25	17·50
Once-washed	1	4·00	4·00	4·00	3·25	2·50	2·50	20·25
	2	4·00	3·25	3·25	2·50	2·50	2·25	17·75
	3	3·00	2·50	2·50	2·25	1·50	0·75	12·50
	4	3·50	3·50	2·25	2·50	2·50	2·25	17·50
Mean		3·75	3·25	3·25	2·75	2·25	2·00	17·00
Twice-washed	1	3·75	3·50	3·50	2·50	2·25	2·25	17·75
	2	4·00	3·25	3·25	2·50	2·50	2·25	17·75
	3	3·25	2·50	2·50	2·25	1·25	0·75	12·50
	4	3·50	3·50	3·25	2·50	2·50	2·25	17·50
Mean		3·75	3·20	3·25	3·00	2·25	2·00	16·50

Table 4. *Summary of the analyses of variance for the motility data in Tables* 1, 2 *and* 3, *showing variance ratios with the error (interaction) mean square in italics at the base of the columns*

Source of variation	Ram				Bull		Rabbit	
	D.F	Variance ratio	D.F.	Variance ratio	D.F.	Variance ratio	D.F.	Variance ratio
Between treatments	—	—	—	—	2	1·3	2	1·9
Unwashed *v.* once-washed	1	0·2	—	—	1	0·5	1	0·7
Unwashed *v.* twice-washed	—	—	1	10·4*	1	0·8	1	3·7
Between ejaculates	3	19·3*	7	5·6*	3	2·3	3	31·1**
Interaction (error)	3	*31*	7	*135*	6	*146*	6	*11*

* $P<0.05$. ** $P<0.01$.

Table 5. *Hourly oxygen uptake in microlitres* (ZO_2) *of* 10^8 *ram spermatozoa incubated in phosphate-fructose diluent at* 37° C. *and* pH 7·0 *for* 5 *hr., showing also in some instances direct bacterial counts on the flask contents at the end of this period*

Treatment	Ejaculate	ZO_2					Total oxygen uptake	Bacterial count 10^6 cells/flask
		1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.		
Unwashed	1	13·8	11·1	13·9	24·8	33·1	96·7	—
	2	16·9	12·1	11·0	12·6	12·7	65·3	—
	3	9·6	7·8	10·8	12·0	9·8	50·0	—
	4	10·5	8·2	11·6	13·8	18·2	62·3	—
	5	11·4	10·3	9·5	9·0	9·3	49·5	69
	6	14·2	11·4	9·6	9·2	6·1	50·5	227
	7	16·5	10·9	9·8	10·1	12·1	59·4	299
	8	15·1	7·3	6·9	6·4	6·9	42·6	293
Mean		13·5	9·9	10·4	12·2	13·5	59·5	111
Once-washed	1	16·3	12·5	9·0	18·0	12·5	68·3	—
	2	17·6	11·4	7·9	8·4	9·0	54·3	—
	3	11·4	7·6	8·7	7·3	5·4	40·4	—
	4	9·6	7·5	9·0	11·3	19·7	57·1	—
Mean		13·7	9·8	8·7	11·3	11·7	55·0	—
Twice-washed	1	19·6	11·4	5·9	8·3	6·5	51·7	—
	2	14·3	9·6	8·9	7·4	9·4	49·6	—
	3	8·9	6·7	6·5	5·8	4·1	32·0	—
	4	10·1	8·0	8·9	10·4	16·1	53·5	—
	5	11·4	7·5	6·4	5·8	4·1	35·2	39
	6	13·5	11·2	7·8	6·1	4·0	42·6	102
	7	14·8	11·5	9·5	6·6	4·8	47·2	119
	8	17·2	10·0	6·0	4·2	2·7	40·1	106
Mean		13·7	9·5	7·5	6·8	6·5	44·0	92

Table 6. *Hourly oxygen uptake in microlitres* (ZO_2) *of* 10^8 *bull spermatozoa incubated in fructose-phosphate diluents at* 37° C. *and* pH 7·0 *for* 5 *hr.*

Treatment	Ejaculate	ZO_2					Total oxygen uptake
		1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	
Unwashed	1	15·8	9·0	6·4	3·5	3·0	37·7
	2	8·5	5·9	5·7	4·1	3·5	27·7
	3	15·7	12·9	11·5	9·1	6·6	55·8
	4	12·0	10·2	9·8	8·4	7·7	48·1
Mean		13·0	9·5	8·4	6·3	5·2	42·3
Once-washed	1	14·3	10·2	8·2	5·0	2·8	40·5
	2	9·5	7·3	7·3	4·2	3·7	32·0
	3	15·6	12·1	12·2	10·7	8·7	59·3
	4	12·6	10·9	10·6	11·4	10·9	56·4
Mean		13·0	10·1	9·6	7·8	6·5	47·1
Twice-washed	1	15·7	7·6	8·4	4·2	3·2	39·1
	2	8·4	6·2	4·3	2·8	2·9	24·6
	3	18·9	13·2	10·9	10·8	11·0	64·8
	4	12·3	10·4	9·1	8·4	7·6	47·8
Mean		13·8	9·4	8·2	6·6	6·2	44·1

Oxygen uptake

Tables 5, 6 and 7 give the hourly oxygen uptake of ram, bull and rabbit spermatozoa respectively and Table 8 the summary of the analyses of variance.

Ram. Washing once had no significant effect, but washing twice reduced the total oxygen consumption ($P < 0.01$). Ejaculates did not vary significantly.

Bull. No significant effect was seen on washing, but highly significant variation occurred between ejaculates.

Table 7. *Hourly oxygen uptake in microlitres* (ZO_2) *of* 10^8 *rabbit spermatozoa incubated in fructose-phosphate diluent at* 37° C. *and* pH 7·0 *for* 5 *hr.*

Treatment	Ejaculate	ZO_2					Total oxygen uptake
		1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	
Unwashed	1	4·6	4·6	3·8	6·9	14·7	34·6
	2	8·0	7·0	6·4	5·0	4·0	30·4
	3	6·0	6·0	6·0	4·2	3·6	25·8
	4	10·0	5·9	7·2	6·5	5·6	35·2
Mean		7·2	5·9	5·9	5·7	7·0	31·5
Once-washed	1	5·7	5·2	4·5	5·0	2·8	23·2
	2	8·4	5·4	6·6	3·5	2·9	26·8
	3	7·1	4·9	4·9	3·0	2·2	22·1
	4	8·0	4·0	5·7	5·4	3·7	26·8
Mean		7·3	4·9	5·4	4·2	2·9	24·7
Twice-washed	1	4·7	3·5	3·3	3·0	2·0	16·5
	2	7·4	6·8	3·4	3·4	3·4	24·4
	3	8·9	4·9	4·9	2·1	3·9	24·7
	4	6·5	4·5	4·1	4·1	3·2	22·4
Mean		6·9	4·9	3·9	3·2	3·1	22·0

Table 8. *Summary of the analyses of variance for the oxygen uptake data in Tables* 5, 6 *and* 7, *showing variance ratios with the error (interaction) mean square in italics at the base of the columns, the data have been coded by multiplying by ten*

Source of variation	Ram				Bull		Rabbit	
	D.F.	Variance ratio	D.F.	Variance ratio	D.F.	Variance ratio	D.F.	Variance ratio
Between treatments	—	—	—	—	2	1·8	2	6·7*
Unwashed *v.* once-washed	1	7·1	—	—	1	3·6	1	6·4*
Unwashed *v.* twice-washed	—	—	1	11·7**	1	0·5	1	12·6*
Between ejaculates	3	9·1	7	3·2	3	46·3**	3	0·8
Interaction (error)	3	*5206*	7	*8289*	6	*1245*	6	*1435*

* $P < 0.05$. ** $P < 0.01$.

Rabbit. Results for both the once- and twice-washed groups were each less than the unwashed control ($P < 0.05$). No significant variation occurred between ejaculates.

Lactic acid production

Table 9 gives the lactic acid production of ram, bull and rabbit spermatozoa during a 5 hr. period and Table 10 the summary of the analyses of variance.

Ram. Washing once had no significant effect, but washing twice reduced lactic

acid production ($P < 0{\cdot}01$). Significant variation occurred between ejaculates in the experiments on twice-washed ram spermatozoa but not in those on once-washed suspensions.

Bull. Again the production of lactic acid was decreased on washing twice ($P < 0{\cdot}01$) but not after a single washing. Highly significant variation occurred between ejaculates.

Table 9. *Micrograms of lactic acid produced by* 10^8 *spermatozoa incubated aerobically in fructose-phosphate diluent at* 37° C. *and* pH 7·0 *for* 5 *hr.*

Treatment	Ejaculate	Ram	Bull	Rabbit
Unwashed	1	191	590	339
	2	238	301	412
	3	408	627	487
	4	303	238	412
	5	187	—	—
	6	225	—	—
	7	459	—	—
	8	363	—	—
Mean		297	439	413
Once-washed	1	117	541	287
	2	261	335	306
	3	309	696	270
	4	229	193	392
Mean		229	441	314
Twice-washed	1	77	335	198
	2	99	208	361
	3	132	467	195
	4	84	48	269
	5	155	—	—
	6	211	—	—
	7	339	—	—
	8	196	—	—
Mean		161	270	256

Table 10. *Summary of the analyses of variance for the lactic acid production data in Table* 9, *showing variance ratios with the error (interaction) mean square in italics at the base of the columns*

Source of variation	Ram				Bull		Rabbit	
	D.F.	Variance ratio	D.F.	Variance ratio	D.F.	Variance ratio	D.F.	Variance ratio
Between treatments	—	—	—	—	2	25·7**	2	4·5
Unwashed *v.* once-washed	1	4·3	—	—	1	0·0	1	4·7
Unwashed *v.* twice-washed	—	—	1	19·0**	1	38·0**	1	8·3*
Between ejaculates	3	9·6*	7	3·8	3	78·5**	3	1·8
Interaction (error)	3	*1456*	7	*3849*	6	*1510*	6	*4185*

* $P < 0{\cdot}05$. ** $P < 0{\cdot}01$.

Rabbit. Although the overall between-treatment variance does not test as significantly different from the interaction mean square, it is nevertheless clear from the analyses that lactic acid production was also depressed on washing rabbit spermatozoa twice ($P < 0{\cdot}05$). Ejaculates did not differ significantly.

DISCUSSION

Comparison of metabolic data

The ZO_2 values obtained in these experiments over the first hour with ram spermatozoa vary from 8·9 to 19·6 with a mean of 13·6 (Table 5), and tend to be lower than those of Lardy, Winchester & Phillips (1945) who recorded values between 11·0 and 46·0. The divergence might be due not only to animal variation but also to differences in diluents, the one used here being much simpler in composition than the Ringer-glucose-phosphate of Lardy & Phillips (1943). It would be interesting, therefore, to study the effect on the oxygen uptake of those ions present in Ringer's solution but absent from the diluent used here.

The ZO_2 (μl. $O_2/10^8$ spermatozoa/hr.) values obtained in these experiments over the first hour with bull spermatozoa vary from 8·4 to 18·9 with a mean of 13·3 (Table 6) and are thus similar to those for the ram. Values recorded in the literature for the ZO_2 of bull spermatozoa vary considerably. Thus Lardy & Phillips (1943) from a study of nineteen samples obtained a ZO_2 range from 16·1 to 29·8, whilst subsequent studies on a large number of other bulls (Ghosh, Casida & Lardy, 1949) gave figures mostly between 3·0 and 10·0, the highest value being 12·6. The cause of the considerable variation in the respiratory activity of bull spermatozoa has not been studied; one possible factor, however, might be differences in the thyroid status of the animals. It has been demonstrated by Schultze & Davis (1948) and confirmed by Maqsood (1950) that the oxygen uptake of bull spermatozoa, at least *in vitro*, is increased by treatment with thyroxine. An extension of this work to include studies of the *in vivo* effect of thyroxine on the oxygen consumption of spermatozoa is clearly desirable.

The ZO_2 values obtained in these experiments over the first hour with rabbit spermatozoa vary from 4·6 to 10·0 with a mean of 7·1 (Table 7) compared with a mean figure of 10·9 reported by Lardy & Phillips (1943) and values between 4 and 28 calculated from the data of Carter (1932).

The mean aerobic lactic acid production [161 μg./5 hr. (Table 9)] by twice-washed ram spermatozoa in these experiments is less than half the values quoted by Mann & Lutwak-Mann (1948), whilst those for unwashed and once-washed bull spermatozoa [439 and 441 μg./5 hr. respectively (Table 9)] are about twice those reported by Henle & Zittle (1942) and Lardy & Phillips (1941). There appear to be no figures in the literature for the lactic acid production of rabbit spermatozoa, but the observations made here indicate that its metabolism, like that of the ram and bull, is highly glycolytic.

Between-ejaculate variation

In these experiments significant between-ejaculate variation occurred in motility, oxygen uptake and lactic acid production. Since pooled ejaculates were used from different combinations of animals on different days it is impossible to assess from the data presented the relative magnitude of day-to-day variation within individual animals compared to day-to-day variation due to the use of different animals on

different days. Other observations made in this laboratory on individual ejaculates, however, indicate that both types of variation in all three species may be considerable so far as motility is concerned. The observations of other workers would indicate that this is also true for oxygen uptake and lactic acid production. Thus Lardy, Winchester & Phillips (1945) working with ram spermatozoa, and Romijn (1950) with bull, record considerable differences in ZO_2, not only between rams but also between samples taken from the same rams on different days, while Comstock's (1939) observations are similar for lactic acid production in the ram.

Hour-to-hour variation

In almost all experiments (Tables 1, 2 and 3) there was a decline in motility during the 5 hr. period in which observations were made; the spermatozoa of all three species were, however, usually quite active at the end.

During the course of most experiments there was also a decline in ZO_2 (Tables 5, 6 and 7), although there was no striking correlation between decline in motility and falling off in oxygen consumption. This is, perhaps, not unexpected, since energy for motility would presumably be available not only from oxidative processes but also from glycolysis—a process which actively takes place in ram, bull and rabbit spermatozoa even under the aerobic conditions of these experiments (Table 9). Further factors mitigating against any close correlation between oxygen uptake and motility are that the motility observations are of a subjective nature and not necessarily linearly related to, or having the precision of, the measurements of oxygen consumption made by the manometric method.

Towards the end of some experiments on the ram (ejaculates 1, 4 and 7 in Table 5) and the rabbit (ejaculate 1 in Table 7) the oxygen consumption rose rather than fell. This was thought to be due either to the proliferation of bacteria with high respiratory activity or else to the liberation of some metabolic regulator possibly related to that of Lardy, Ghosh & Plaut (1949). Bacterial contamination was tested by experiments on unwashed ram spermatozoa in which streptomycin was added at a final concentration of 1 mg./ml. of spermatozoa suspension. Tables 11 and 12 show the results of such an experiment done on an ejaculate in triplicate and Table 13 the analysis of variance. Significant variation in total oxygen uptake occurred between the control and streptomycin-treated groups, and it is clear from the data that the difference is due to the suppression by streptomycin of the rise in ZO_2 seen in the control group during the latter hours of the experiment. This is taken as evidence that the phenomenon is due to bacteria. The streptomycin was innocuous to the spermatozoa, since neither the ZO_2 over the first hour (Table 11) nor the total motility score (Table 12) were significantly affected by it.

Direct bacterial counts (Table 5) made on some flask contents at the end of a 5 hr. run indicated that bacteria were present in large numbers even when there was no very great increase in oxygen consumption. No doubt the type of organisms present is as important as the total bacterial count in determining whether or not the effect on oxygen uptake will occur, since different bacterial species are known

to vary widely in their oxygen requirements. The sources of bacterial contamination in these experiments are multiple and could include the urogenital tract of the animal, the artificial vagina, the glassware and the diluents. Since it is virtually impossible to achieve asepsis in these experiments, it would appear unwise to prolong them beyond 3 hr., which is in keeping with the experience of Tosic &

Table 11. *Hourly oxygen uptake in microlitres* (ZO_2) *of* 10^8 *unwashed ram spermatozoa incubated in fructose-phosphate diluent at* 37° C. *and* pH 7·0 *with and without* 3 *mg. of streptomycin per flask*

Treatment	ZO_2					Total oxygen uptake
	1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	
Control	10·2	8·5	13·2	16·8	24·5	73·3
	10·9	8·7	14·0	20·6	22·1	76·3
	9·8	8·1	11·3	21·9	25·7	76·8
Streptomycin	9·4	6·6	7·4	11·3	8·9	43·6
	9·8	7·2	6·8	8·9	10·4	44·1
	9·8	8·3	10·2	13·0	6·0	47·3

Table 12. *Lactic acid production and total motility score of unwashed ram spermatozoa incubated for* 5 *hr. in fructose-phosphate diluent at* 37° C. *and* pH 7·0 *with and without* 3 *mg. of streptomycin per flask*

Treatment	Lactic acid (μg./10^8 cells)	Total motility score
Control	344	15·5
	350	18·5
	376	19·5
Streptomycin	363	17·0
	328	15·5
	318	19·0

Table 13. *Summary of the analyses of variance for the data in Tables* 11 *and* 12, *showing variance ratios with the error mean square in italics at the base of the columns*

Source of variation	D.F.	Oxygen uptake	Lactic acid production	Motility
Between treatments	1	354·4**	1·5	0·2
Remainder	4	*392*	*424*	*371*

** $P<0.01$.

Walton (1950). The first 3 hr. probably represent a fairly true estimate of the respiration of the actual spermatozoa; thereafter the results are likely to be complicated by bacterial growth making analysis and interpretation more difficult. Tyler & Rothschild (1951) in prolonged experiments on sea-urchin spermatozoa encountered a similar increase in oxygen uptake which, by the use of penicillin, they showed was due to the proliferation of bacteria in the sea water.

Effect of washing

The results reported here show that the spermatozoa of the ram, bull and rabbit can be washed once without any significant decrease in motility, but that on washing twice motility may be adversely affected. These findings are in agreement with those of Mann (1945), who states that, as a rule, the washing of spermatozoa should not be repeated more than once, although he does not give any data nor explain the nature of the damage observed.

Tables 5 and 7 indicate that the significant variation in total oxygen uptake seen between washings for the ram and rabbit (Table 8) is mainly due to increases in the ZO_2 occurring in the unwashed group in the latter stages of some experiments. The effect of washing is, in fact, somewhat similar to that of streptomycin, i.e. it tends to reduce the terminal rise in oxygen consumption without influencing the initial oxygen uptake. This is in keeping with the belief that the terminal increase in oxygen uptake is due to bacteria, since washing significantly reduces the bacterial count of the spermatozoa suspensions (Tables 5 and 14). The fact that washing had

Table 14. *Analysis of variance for the bacterial counts* (*Table* 5) *on unwashed and twice-washed ram semen incubated for* 5 *hr. in fructose-phosphate buffer at* 37° C. *and* pH 7·0

Source of variation	D.F.	Sum of squares	Mean square	Variance ratio
Between treatments	1	34,050	34,050	12·9*
Between ejaculates	3	30,350	10,117	3·8
Interaction (error)	3	7,897	2,632	—

* $P < 0.05$.

little, or no, effect on oxygen uptake during the early hours of the experiment suggests that the respiration of the spermatozoa themselves was not affected by this treatment.

Whilst the effect of washing on oxygen consumption in these experiments is most probably dependent upon washing out bacteria, the significant reduction in lactic acid production seen in the ram, bull and rabbit (Table 10) on washing is believed to be a direct effect of the spermatozoa. That this is so is indicated by the streptomycin experiment (Table 12) in which no significant difference in lactic acid production is seen between the control and the antibiotic-treated group (Table 13). This evidence has been added to in other experiments in this laboratory on ram and bull spermatozoa, in which it has been shown that washing significantly reduces lactic acid production during the first hour of incubation, when bacteria do not materially influence the results.

The decreased production of lactic acid on washing spermatozoa is in accordance with the classical observations on the ease with which the glycolytic components of tissues and cells, e.g. muscle and yeast, can be extracted in aqueous solution (Baldwin, 1949). The nature of the important components of the glycolytic cycle which might be removed in the washing of spermatozoa is a matter for speculation.

If they are the less complex ones, e.g. ions or coenzymes, then it should be possible to prevent the adverse effect of washing on glycolysis by including these substances in the diluent. If, on the other hand, the washing involves the loss of substances of high molecular weight, e.g. enzymes, the process might be more difficult to prevent. It should be noted in this regard that large molecules such as hyaluronidase (Swyer, 1947) and cytochrome *c* (Mann, 1951) are known to pass readily from the spermatozoan cell.

The relationship between the repetitive washing and the dilution of spermatozoa has been discussed by Emmens & Swyer (1948) who concluded that they are analogous phenomena. If this is so then one might expect from these results to find a loss of glycolytic power on severe dilution of spermatozoa and, as with the washing effect, to prevent it by the fortification of the diluting medium with those components lost from the cell. The effectiveness of such diverse agents as gum arabic, starch, glycogen and serum proteins in mitigating the effect of dilution (Emmens & Swyer, 1948) might then be to prevent the leakage of the glycolytic components from the spermatozoa.

Work being undertaken in this laboratory to elucidate the nature of the essential substances leached from spermatozoa on washing and dilution would indicate that potassium is at least one important factor.

SUMMARY

1. The motility, oxygen uptake and aerobic glycolysis of unwashed, once- and twice-washed ram, bull and rabbit spermatozoa have been studied in a sodium phosphate-fructose diluent over a 5 hr. period at 37° C.

2. The mean ZO_2 values obtained over the first hour for ram, bull and rabbit spermatozoa were 13·6, 13·3 and 7·1 respectively, and the corresponding mean total lactic acid production for each of these species over the 5 hr. period was 297, 439 and 413 μg./10^8 cells. Significant differences in oxygen uptake, lactic acid production and motility occurred between pooled ejaculates.

3. There was a decline in motility in almost all experiments, and a similar decline in oxygen consumption during the early hours. Towards the end of some experiments on unwashed ram and rabbit spermatozoa there was a rise in oxygen uptake which was shown to be due to bacterial contamination.

4. Washing once had no significant effect on motility, but washing twice adversely affected the motility of ram spermatozoa.

5. Significant decreases in total oxygen uptake occurred on washing ram spermatozoa twice and on washing rabbit spermatozoa both once and twice. This is believed to be due to the removal of bacteria.

6. Washing once had no significant effect on total lactic acid production, but it was significantly reduced on washing ram, bull and rabbit spermatozoa twice. This effect is believed to be associated with the spermatozoa themselves.

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THE GREGARIOUS BEHAVIOUR OF MARCHING *LOCUSTA MIGRATORIA MIGRATORIOIDES* (R. & F.) HOPPERS

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INTRODUCTION

Locusta migratoria migratorioides (R. & F.) is a highly polymorphic species, the differences in anatomy and colour being so great that the extremes were at one time classed as separate species. Variations in behaviour are also striking and for the nymphs or hoppers seem to be related to the density of the population, although this may not be the only factor operating. There are practically no published observations on the behaviour of *L. migratoria migratorioides* in the field. Information on other locusts has been freely drawn upon, although the behaviour of the various species is probably not identical.

The evidence at present available suggests that, when populations are thin, the hoppers live in relative isolation and spend most of the day basking on the ground or resting in the plants. When hopper populations are dense, the individuals live in groups or bands and carry out a characteristic daytime wandering called marching. Locusts tend to live in relatively uncultivated country, so that their ability to emigrate from these areas into others of intensive cultivation greatly adds to the economic importance of their capacity for very rapid population increases. Although they cannot move as rapidly or as far as adults, yet hopper bands (which may cover many acres) can severely damage crops when they march through cultivated fields. In addition to its economic importance marching behaviour is, like all mass movements of animals, of considerable biological interest.

The individual hoppers of a marching band tend to move in the same general direction, progressing by periods of steady walking, interspersed with long low hops. It appears that in other species of locusts, the hoppers from such bands are far less active when alone (Fraenkel, 1929; Kennedy, 1939, on *Schistocerca gregaria* (Forsk.)). Clark (1949) gave a good example of the increased marching of large bands. On one particular day a large and a small band of *Chortoicetes terminifera* (Walker) were wandering in the same direction, fairly close to one another. The large band travelled 45 ft., but the small band only 6 ft. in 1 day. Uvarov (1928) suggested that hoppers in groups were more active than isolated ones, because of an opto-motor reaction: when one hopper moved those near it also moved in such a way as to keep the visual field constant. Field studies have reaffirmed the theoretical importance of opto-motor reactions, although it is clear that other kinds of hopper interaction also play a part (Volkonsky, 1942; Kennedy, 1945, 1951; Clark, 1949).

The optimal conditions for the marching of *Locusta migratoria migratorioides* in the laboratory have been studied in detail (Ellis, 1951). A group of hoppers will march round and round in a cage, provided there is a single source of light overhead. It is easy to recognize a marching hopper, for the body is carried in a characteristic way with the frons perpendicular to the ground, the antennae erect and the

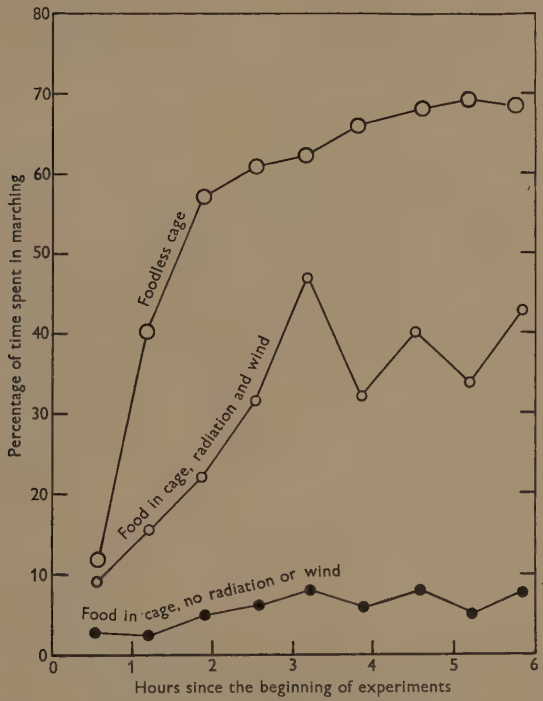

Fig. 1. Marching activity in relation to air currents (wind) plus radiant heat, and starvation. Air temperature 32° C.; 80 4th-instar hoppers per 30 × 30 by 25 cm. high cage. Each curve represents the average for three separate tests. Radiation plus wind caused an increase in marching, whilst starvation caused an even greater increase.

whole body kept well clear of the ground. Marching is most vigorous and prolonged at temperatures between 29 and 35° C., being greatly increased by starvation for 3–13 hr. With fresh grass present it requires the combined effects of radiant heat and air currents, factors which are normally present in the field (Fig. 1).

Even with other facts optimal, marching varies with the number of hoppers per cage. As marching is favoured by 'mild' physical conditions, it has been suggested that it is a normal activity of group-living hoppers, which takes place whenever it is not suppressed by an incompatible activity (Ellis, 1951). A similar conclusion

was reached by Kennedy (1939, 1951), after studying *Schistocerca gregaria* in the field. This paper analyses further the problem of hopper numbers and marching, and especially the hopper interactions responsible for the increase in activity with numbers. Only hoppers reared in crowds for many generations were used. Such hoppers are generally said to belong to the phase *gregaria*, but this term will be avoided, as it has been used to describe locusts with certain anatomical ratios and colours, which are often, but not always, associated with locusts that are living in groups (Key, 1950; Gunn & Hunter-Jones, 1952). The behaviour of hoppers reared in isolation, so-called *solitaria*, will be dealt with in a later paper.

EXPERIMENTAL ANIMALS

The experimental animals were 4th instar hoppers of *Locusta migratoria migratorioides* (R. & F.), bred at the Anti-locust Research Centre, London. The same stock was studied by Hamilton (1936), Norris (1950) and Ellis (1951). The hoppers were reared in a constant temperature room which was maintained at 30–31° C. during the tests on hopper numbers and densities, and at 27–28° C. during the rest of the study. The hoppers were kept under crowded conditions of 300–400 1st instar nymphs in a cage 43 × 43 by 25 cm. high; by the 4th instar 200–250 were still alive. Each evening they were fed with grass standing in water, most of which had been eaten by the following morning. During the day a 60 W. lamp was hung centrally above each cage and the hoppers were allowed to march for at least 8 hr. When the room was maintained at 30–31° C., the lamp was water-screened to prevent excessive heating (Fig. 2). Sticks and hay were placed in the corners of the cage to provide moulting perches.

The test hoppers had moulted 2–3 days before use: during the 4th instar, activity is depressed after the 4th day, as moulting time approaches (Ellis, 1951). Individuals were identified by spots of poster paint on the pronotum and abdomen. During the night preceding a test the hoppers were well fed, being starved next morning for various periods before use. When they were needed in a relatively inactive state they were starved for 1 hr., and in an active state for 3 hr. (Fig. 1). During starvation they were placed in jars arranged round an electric lamp. Additional unmarked hoppers were used in some of the tests; they were the same age as the marked hoppers and were previously well fed: they will be called background hoppers.

MARCHING VIGOUR IN RELATION TO HOPPER NUMBERS AND DENSITY

Apparatus and test conditions

During these tests three sizes of cage were used; they were all 25 cm. high and the floor areas were 30 cm. square, 43 cm. square and 60 cm. square. The floors and walls were made of fibre-board and the tops of glass with a triangular gauze lid in one corner. During tests the cages were kept clear of obstructions and food, a 60 W. lamp was hung centrally above each and a water screen (to prevent excessive

heating) was placed between the lamp and the cage top. The lamp and water dish were surrounded by a cylindrical black paper shade (Fig. 2). Observations were made through the glass top of the cage, but did not disturb the hoppers inside, provided the lamp was screened and the rest of the room was kept in darkness.

At the beginning of a test the hoppers were transferred from the feeding cages to the experimental cages. The numbers of hoppers per cage were 2, 15, 30, 50 and 100, the test at each number being repeated three times. Five hoppers were marked in

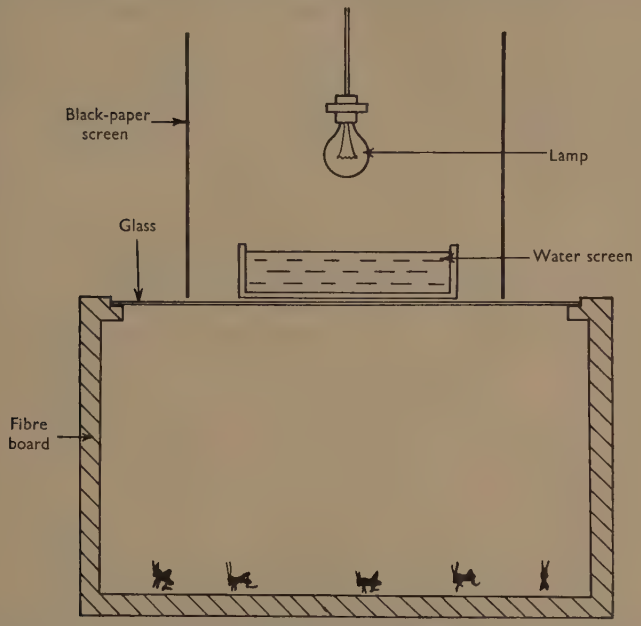

Fig. 2. Diagram showing the arrangement of cage lighting for tests carried out in square cages at a temperature of 30–32° C.

each cage (in experiments with only two hoppers, both were marked) and during the 6 hr. test they were observed for 10 consecutive min. per $\frac{1}{2}$ hr. At each reading it was noted whether the marked hoppers were marching or not, and from these readings the percentage of time spent in marching was calculated. Observations which gave the actual speed of the hoppers whilst marching (marching speed) were also made, but will not be considered further. The smaller the cage the lower was the marching speed, suggesting that the hoppers were slowed down by having to travel in small circles. A few tests with one hopper per cage were carried out, continuous observations being made for 1 hr., after they had been starved for 5 hr.

Results

In Fig. 3 the average percentage of time spent in marching for each number of hoppers per cage was plotted against time, there being a separate curve for each cage. It has been shown that the rise in marching activity during tests in foodless cages is related to the emptying of the gut and to a cumulative effect of hopper interactions (Ellis, 1951). On the whole, the curves for each cage were similar with 30 or more hoppers present, but with smaller numbers there was less activity the larger the cage. Thirty per cent of the time was spent in marching after 5 hr. of

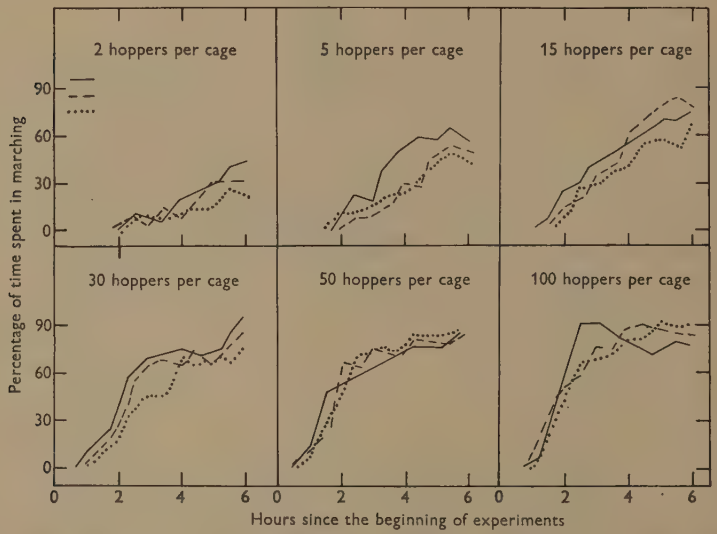

Fig. 3. Marching activity in relation to time since the beginning of starvation, for 4th-instar hoppers in cages of three different sizes. Temperature 30–31° C. Numbers of hoppers per cage varied from 2 to 100. Each curve represents the average for three separate tests. The onset of marching was delayed with 30 or fewer hoppers and maxima were reduced with two and five. With fewer than 15 hoppers marching was greatest in the smaller cages.

starvation with two hoppers per cage, after 4 hr. with five hoppers, after $3\frac{1}{2}$ hr. with 15 hoppers, after $2\frac{1}{2}$ hr. with 30 hoppers, after 2 hr. with 50 hoppers and after $1\frac{1}{2}$ hr. with 100 hoppers. That is, the onset of marching was significantly delayed with fewer than 30 hoppers present. With only two and five the maxima were also lower, although it is possible that with longer periods of starvation all maxima would be the same. Ten hoppers were tested by themselves. Two of them did not march during the observation period, whilst three of them marched for over 70% of the time. The average was 32%, a value very similar to that for two hoppers per cage after the same period of starvation, namely 5 hr.

In order to test the relative importance of the total number of hoppers present and the cage size, the readings for each 6 hr. test were averaged. An analysis of variance on the results showed that numbers significantly affected marching activity ($P<0{\cdot}01$), whilst the importance of cage size was doubtful ($P=0{\cdot}02$–$0{\cdot}05$). Examining the actual means in the light of this analysis, the overall mean for the 60 cm. cage was significantly lower than that for the 30 cm. one. The overall means for the numbers of hoppers per cage differed significantly for each increase up to 50: there was no significant difference when the results for 50 and 100 hoppers

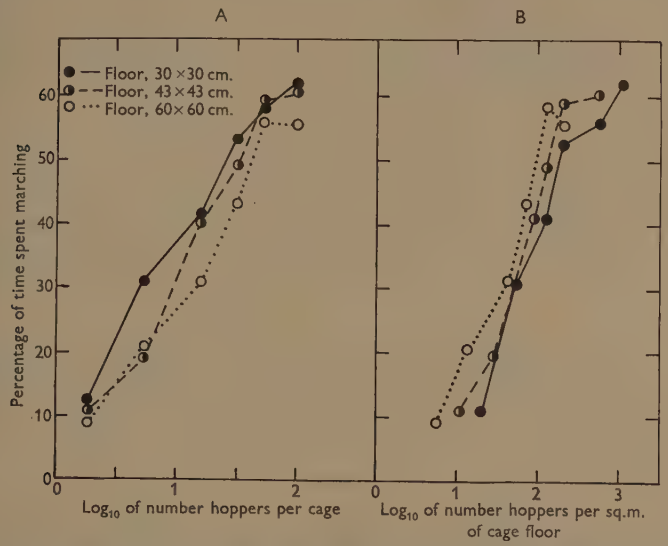

Fig. 4. The relationship between the average percentage of time spent marching for whole 6 hr. tests and (A), the total numbers of hoppers per cage and (B), the numbers of hoppers per square metre of cage floor (area-density). Temperature 30–31° C., cages foodless and hoppers in the 4th instar. Each point represents the average for three tests. With 50 or more hoppers per cage marching depended on numbers, but below 50 it depended on both numbers and area-density. In all cages, marching was reduced with fewer than 30 hoppers present.

were compared. A similar conclusion is to be drawn from Fig. 4 in which the percentage of time spent marching was plotted against (A), the $\log_{10}$ of the total number of hoppers present and (B), the $\log_{10}$ of the number of hoppers per square metre of cage floor, called area-density for short. Logarithms were used in order to foreshorten the curves at the higher numbers and area-densities, where activity is unaffected by numbers. With a given number of hoppers present, marching was greater in the small cages (Fig. 4A), but with a given area-density marching was greater in the large cages (Fig. 4B). This again shows that numbers and area-density were important, although with more than 50 hoppers per cage marching may have depended on numbers rather than area-density.

These tests clearly showed that vigorous marching was a group activity, which was greater in large and closely packed hoppers groups than in small and more scattered ones. The rest of this paper deals with the hopper interactions which lead to this increased marching activity.

THE ANTENNAE AND MARCHING ACTIVITY

When marching is well established the antennae are kept still and seem rarely to be used, but at the beginning of marching, when hoppers spend some time wandering about at random, the antennae are continually used to examine the floor and other hoppers. A resting hopper examined by an active one, kicks with its hindlegs if touched on the abdomen, but twirls its antennae in a face-to-face encounter. These reactions generally result in the active hopper moving away.

Apparatus and test conditions

The marching activity of antenna-less, 4th-instar hoppers was compared with that of normal individuals. The experimental hoppers were marked in the usual way and the tests were carried out in cages 43×43 by 25 cm. high, made of fibre-board, but with a glass top. A 60 W. lamp, water-screen and cylindrical shade were placed centrally above the cage (Fig. 2). The room temperature was maintained at 30–31° C.

The 16 marked hoppers and 40 background hoppers were well fed overnight and were placed in the test cage next morning. During the next 6 hr. marching by the marked hoppers was noted once per min., for 10 consecutive min. per $\frac{1}{2}$ hr.; from these readings the percentage of time spent marching was calculated. The complete test was repeated 3 times.

Results

The results for the eight antenna-less hoppers were averaged for each day and compared with the corresponding averages for the eight control hoppers. These averages are shown in Fig. 5, in which the percentage of time spent marching was plotted against time. The two curves represent the means for the three tests. Amputation of antennae had no marked effect on marching, although the graph suggests that the antenna-less hoppers were a little more active during the first 3 hr. of test, than the normal hoppers.

THE GENERAL IMPORTANCE OF OPTICAL STIMULI DURING MARCHING

Method

The marching behaviour of 4th-instar hoppers which had had their eyes painted over with poster paint for 20 hr. was compared with that of normal animals. Both kinds of hopper were marked and starved for 5 hr. before being dropped into a cage containing a small marching band of hoppers. A water-screened lamp was placed centrally above the cage (Fig. 2) and the air temperature was maintained at 30–31° C. The test hoppers were observed once per minute during the hour after dropping in.

Activity was classified as marching, pottering (random movement) or resting, and the percentage of time spent in each of these activities was calculated from the 1 min. readings.

At the end of the tests the blinded hoppers were examined with a hand-lens, and any with defective eye coverings were excluded from the final results: four out of 32 hoppers had to be discarded. The complete experiment was repeated on four different days.

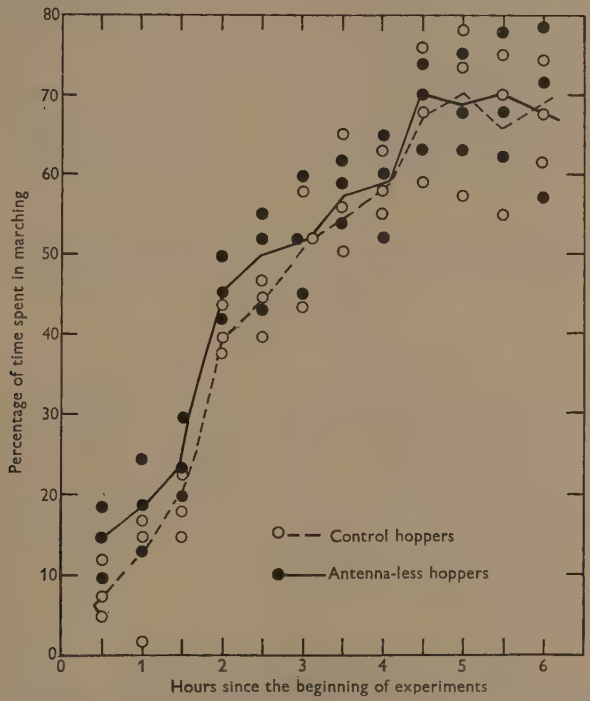

Fig. 5. The marching activity of 4th-instar hoppers with and without antennae. Temperature 30–31° C., cages foodless. The circles represent results for individual tests and the curves the averages for all tests. Amputation of antennae had little effect on marching.

Results

Three of the discarded hoppers performed circling movements for long periods, turning towards the eye which was partially uncovered. Total blinding did not prevent hoppers feeding. On one occasion a blinded hopper rapidly joined with others in devouring a newly moulted fellow and at the end of the tests, when grass was put into the cage, the blinded hoppers were soon feeding with the rest. Presumably, the blinded hoppers moved towards the food in response to olfactory or auditory (sound of other hoppers chewing) stimuli (cf. Volkonsky, 1942).

Twenty-four normal hoppers were tested, and all of them spent some time in marching, the individual results varying between 42 and 83%. Only 58% of the blinded hoppers marched, and for only 8–20% of the time. Generally they appeared to be kept moving in the direction of the general stream by repeated impacts from other hoppers, but four blinded individuals did march for short periods without being pushed. The normal hoppers spent 10–28% of the time pottering, whilst the blinded hoppers spent 2–20% of the time in this activity. The 42% blinded hoppers that did not march went to the cage sides; half of them remained still throughout the test period, whilst the other half averaged three changes in position and pottered for up to 10% of the time.

Although it is tempting to suppose that these experiments suggested the importance of optical stimuli between hoppers in increasing marching, the eyes may nevertheless act as 'stimulationsorgane', so that stimuli received via them lead to a general increase in locomotor activity (Wolsky, 1933; Chauvin, 1947).

AN ANALYSIS OF THE OPTICAL AND MECHANICAL INTERACTIONS BETWEEN HOPPERS WHICH LEAD TO INCREASED MARCHING ACTIVITY

Experimental arrangements

The parts played by optical interactions and physical contact between hoppers (called mechanical interactions for short) in increasing marching activity were tested in a series of five experimental arrangements that eliminated one factor at a time. Three types of experimental cage were used, each containing a circular, ring-like test gallery, 8 cm. high, 8 cm. across, with an inner diameter of 28 cm. and an outer one of 44 cm. The floors were made of fibre-board and were marked into eight equal divisions by pencil marks. The tops and sides were made of celluloid.

(1) *The single gallery cage* consisted of a test gallery with the outer walls backed by white cardboard (Fig. 6A).

(2) *The treble gallery cage* consisted of a test gallery surrounded by an outer gallery 5 cm. across and an inner gallery also 5 cm. across (Fig. 6B). The outer walls of this cage were backed with white cardboard.

(3) In *the moving pattern cage* the test gallery was suspended over metal spokes which carried an outer and an inner cylinder of white cardboard (Fig. 6C). The spokes were soldered to a bicycle wheel hub, mounted upright on a heavy wooden base. The cardboard cylinders carried one of two patterns, which were turned by hand, in an anti-clockwise direction, at about 3 r.p.m.

(*a*) *Vertical stripes*. There were 64 alternately black and white stripes, 2·5 cm. wide, on the outer cylinder and 64 stripes, 1 cm. wide, on the inner cylinder.

(*b*) *Dead hoppers*. Thirty-two dead, 4th-instar hoppers were stuck 2·5 cm. apart on the outer cylinder and 18 on the inner cylinder, facing in an anti-clockwise direction. The dead hoppers were prepared as follows. After killing, the soft exoskeleton in front of the thorax was pierced and the body fluids squeezed out. The

hoppers were then filled with warm wax blown from a glass tube. When dry, these dead hoppers retained their rounded form.

During the tests, a 100 W. lamp, surrounded by a white cardboard shade, 50 cm. high and 60 cm. in diameter, was placed 70 cm. above the floor of each cage.

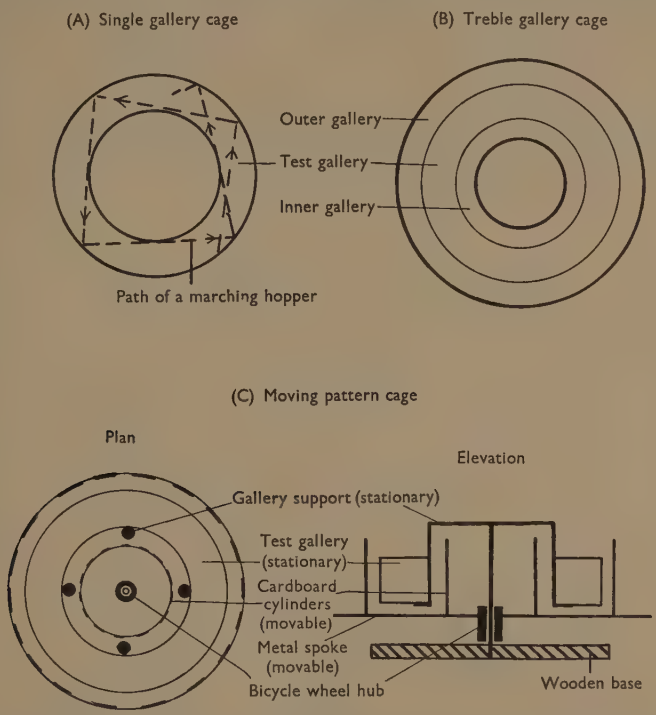

Fig. 6. Diagrams of the cages used in experiments in the mechanical and optical reactions between marching hoppers. Test galleries 8 cm. across, with an inner diameter of 28 cm. and an outer diameter of 44 cm.

The five experimental arrangements were:

(1) *Hopper contacts*. The *single gallery cage* contained 50 marching background hoppers.

(2) *Gallery hoppers*. There were 20 marching background hoppers in the inner gallery and 60 in the outer gallery of the *treble gallery cage*.

(3) *Dead hoppers*. The dead hoppers stuck to cardboard were moved round outside the *moving pattern cage*.

(4) *Vertical stripes*. The vertical stripe pattern was moved round outside the *moving pattern cage*.

(5) The *controls* were of three kinds. The *single* or *treble gallery cages* empty, or the *moving pattern cage* with plain white cardboard cylinders outside.

The differences between pairs of arrangements were as follows. In *hopper contacts* the test individual gave and received both mechanical and optical stimuli from the marching background hoppers in the cage, whereas in *gallery hoppers* there were only optical reactions between the test individual and the hoppers in the outer and inner galleries. Any differences in the activity of the test hopper when placed in these two cages was therefore due to the mechanical interactions between this individual and the background hoppers. In a similar way the other arrangements analysed the optical reactions between hoppers; *gallery* and *dead hoppers* the importance of live hopper movements; and *dead hoppers* and *vertical stripes* the importance of hopper shape plus colour. The *controls* gave the activity of isolated test hoppers not subjected to any special stimuli. The results for all arrangements must be compared with the *controls*.

The experimental readings

The 4th instar experimental hoppers were tested individually, and during the 5 min. observation period the divisions of the gallery floor travelled clockwise and anti-clockwise were counted. The number of seconds that the hopper spent in activity was also recorded, but was difficult to measure accurately. Most of the walking was marching, although some random movement (pottering) took place: no hopper pottered for more than two divisions of the cage floor per reading. Marching hoppers moved fairly continuously and, especially in the *dead hopper* and *gallery hopper* arrangements, tended to keep to a straight path as long as possible (Fig. 6A). In the *vertical stripes* arrangement many hoppers kept close to one side of the gallery and often scrambled up the wall. With *gallery*, *dead hoppers* and *vertical stripes* arrangements, test hoppers frequently looked squarely out of the gallery and pointed their antennae towards the objects outside.

Table 1. *Frequency distribution tables of individual results for active hoppers, placed in various experimental arrangements* (*see p.* 225)

(The figures are percentages and based on results for 18 females and 19 males.)

Experimental arrangement	Seconds active per min.				
	1–10	11–20	21–30	31–40	41–50
Controls	73	17	10	—	—
Vertical stripes	53	18	23	6	—
Dead hoppers	33	28	24	10	5
Gallery hoppers	47	11	11	16	16
Hopper contacts	32	29	13	10	16

	Progress speeds in cm./min.						
	1–28	29–56	57–84	85–112	113–140	141–168	169–196
Controls	90	10	—	—	—	—	—
Vertical stripes	64	12	12	6	6	—	—
Dead hoppers	29	29	19	14	19	—	—
Gallery hoppers	47	21	5	5	11	11	—
Hopper contacts	39	32	4	13	—	66	6

The data were analysed in six ways. First, the percentage of hoppers that moved during the observation period was calculated and called the *percentage active*. Unfortunately, the individual results were not normally distributed, neither did they follow any other distribution that could be analysed statistically (Table 1), so the other five analyses were based on AVERAGES for the ACTIVE hoppers only. As each division of the cage floor was approximately 14 cm. long, the distance in cm. per min. travelled clockwise (C), anti-clockwise (A) and ($A+C$) were calculated

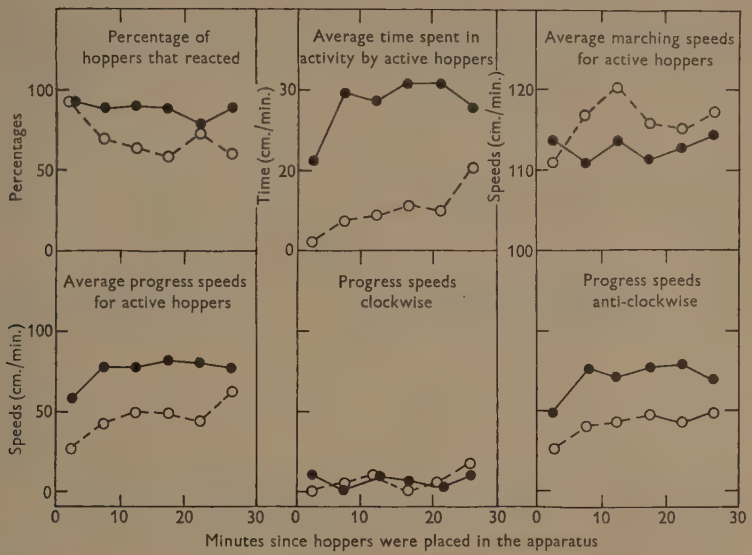

Fig. 7. The marching activity of 4th-instar hoppers in two experimental arrangements. Progress speeds measured distances covered per min. including rests, and marching speeds measured the distance covered per min. excluding rests. Temperature inside the cages 32° C. Hoppers were starved for 1 hr. before testing individually for 30 min. Averages for 9 females and 10 males tested with the vertical striped pattern moved round outside the cage are shown by the open circles and broken lines. Averages for 8 females and 11 males tested with other hoppers marching in the cage are shown by the solid circles and lines. Activity was abnormal during the first 5 min. of test.

separately, by multiplying the divisions of the cage floor travelled per 5 min. reading by 14 and dividing by 5. This measure of activity was called *progress speed* and a change in its value could be due, either to a change in the number of seconds per reading that the hopper was active (the *seconds active*) or to a change in the rate at which the hopper moved whilst actually marching (the *marching speed*). The *seconds active* were obtained by dividing by 5 the total number of seconds active per reading. The *marching speed* was calculated by adding the *progress speeds A* and *C*, irrespective of direction, multiplying the product by 60 and dividing by the *seconds active*, to obtain the result in cm. per min.

Each test hopper was placed in the apparatus for 10 min., observations being made during the second 5 min. During the first 5 min. the hoppers behaved abnormally because of recent handling; that is, the *percentage active* was higher and the *progress speeds* and *seconds active* were generally lower than later on. This is illustrated in Fig. 7, showing the results for tests in which hoppers were observed for 30 min., after being placed in the *hopper contact* or *vertical stripes* arrangements.

Table 2. *The marching activity of isolated 4th-instar hoppers when black and white vertical stripes were moved round outside the cage in an anti-clockwise direction*

(Progress speed measured distance travelled per min including rests, and marching speed measured the distance covered excluding rests. Five tests made per day at 1½ hourly intervals. Results for 7 females and 12 males.)

Number of test	Percentage of active hoppers	Averages for the active hoppers				
		Progress speeds, cm./min.			Sec. active per min.	Marching speed cm./min.
		Clockwise *C*	Anti-clock-wise *A*	*A*+*C*		
1	42·1	2·2	12·9	14·1	4·8	176·2
2	52·6	5·0	20·2	25·2	9·2	164·4
3	57·6	4·8	28·6	33·4	10·2	194·6
4	42·1	5·3	32·5	37·8	14·1	160·9
5	47·6	0	35·3	35·3	14·4	147·1
Largest differences	16	6	23	24	10	48

In order to present the five experimental arrangements to the same individuals in one day, the hoppers were tested after 1 hr. of starvation, fed for 20 min., starved for 1 hr., re-tested and so on. As the test period lasted for 10 min., seven or eight individuals were used in turn. The order in which the five arrangements were presented to the hoppers was altered with each day's test, for even when using the same arrangement (*vertical stripes*) throughout the day, the *progress speeds* and *seconds active* increased with each successive test (Table 2).

Results

When comparing the results for the various arrangements it would be unwise to treat any differences as significant, unless they are greater than those in Table 2: for example, 16 points for the *percentage active* and 10 points for the *seconds active*.

The percentages of hoppers that reacted during the test period were in three groups (Fig. 8), which differed by more than 16 points from one another. Only 30% of the hoppers reacted under *control* conditions, whereas over 80% did so with *hopper contacts*. *Vertical stripes*, *dead* and *gallery hoppers* were intermediate in effect and approximately 50% of the hoppers reacted to them. This means that, even with no special stimuli, some hoppers that have been starved for 1 hr. moved; but more did so in response to a moving background and nearly all of them moved when in actual physical contact with other marching hoppers.

The amount of activity shown by the *active* hoppers was measured by the *progress speeds* $A+C$, the results forming three groups, which differed from those for the *percentages active*. The two extremes, which can be considered to differ significantly, were the *controls* and the *dead hoppers* plus *gallery hoppers* plus *hopper contacts*. The results for the *vertical stripes* were intermediate between the two extreme groups (Fig. 8). Thus, *progress speeds* were increased when the hoppers

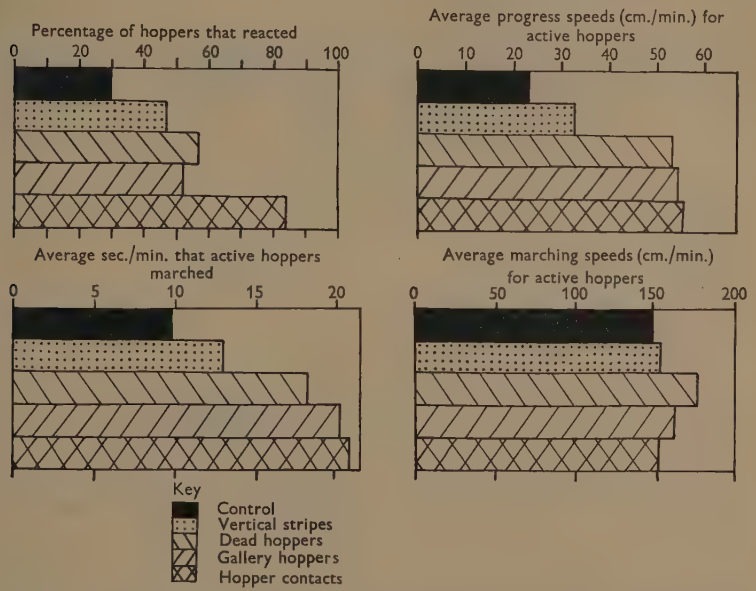

Fig. 8. The marching activity of 4th-instar hoppers in five experimental arrangements (see p. 223). Progress speeds measured distances covered per min. including rests, and marching speeds distances covered per min. excluding rests. Temperature inside the cages 32° C. Hoppers starved for 1 hr. before testing individually for 10 min., and each hopper presented with the five arrangements in one day. Results for 18 females and 19 males. The highest percentage of hoppers gave reactions with *hopper contacts*, the lowest under *control conditions*; the other three arrangements were intermediate. Progress speeds and seconds active were lowest in the *controls*, intermediate with *vertical stripes* and highest with the other three arrangements. Marching speeds did not vary significantly.

were subjected to a moving background of stripes, but the maximal amount of activity was only reached in response to moving hopper bodies, alive or dead. Physical contact between hoppers did not increase *progress speeds* further. It has already been pointed out that the *progress speed* depended on the *seconds active* and the *marching speed*. The results for the *seconds active* were similar to those for the *progress speed* $A+C$, but the *marching speeds* did not vary a great deal as between the various arrangements, although the *controls* gave the lowest and the *dead hoppers* the highest results. It therefore appears that the differences in the *progress*

speeds A + C depend on the amount of time per reading that the hoppers spent in activity, rather than the rate at which they moved when actually marching.

Frequency distribution tables of *progress speed A + C* and *seconds active* (Table 1) show that the average increases in these measures depend on a decrease in the percentage of hoppers moving short distances and an increase in the percentage moving long distances. The results in these tables again suggest the three groups of the *controls*, *vertical stripes*, and *gallery hoppers* plus *dead hoppers* plus *hopper contacts*.

Table 3. *The average distances travelled per min. including rests, in a clockwise (C) and an anti-clockwise (A) direction by active 4th-instar hoppers, placed in various experimental arrangements* (see p. 223)

(Results are for 11 to 31 hoppers.)

Experimental arrangement	Progress speeds in cm./min.		Ratio of progress speeds C : A
	C	A	
Control	10·4	13·2	1·0 : 1·3
Vertical stripes	2·8	29·4	1·0 : 10·5
Dead hoppers	7·6	45·6	1·0 : 6·0
Gallery hoppers	3·9	50·7	1·0 : 13·0
Hopper contacts	11·8	43·7	1·0 : 3·7

The average *progress speeds* travelled in both directions are shown in Table 3. Variations in the *progress speeds A* were similar to those for the *progress speeds A + C* (Fig. 8). *Progress speeds C* showed no clear trend, but were interesting when compared with the *progress speeds A*. The ratios between these two measures (Table 3) suggest that moving test hoppers had little preference for direction under *control* conditions, but that they showed a definite preference for the direction in which visible objects moved. Not only did a moving background increase marching activity, but it caused marching to be in a certain direction. The relative increase in the counter direction walking with *hopper contacts* is not easy to explain.

The effect of air and ground vibrations from other hoppers on marching

Marching hoppers in a cage are clearly audible to the human ear, most of the noise being due to the movement of the hoppers' feet. In common with other insects, locusts respond to a wide range of sounds (Pumphrey, 1940), and Kennedy (1951) suggested that the noise made by moving locusts helps to increase the total activity of the group. Although the last experiment showed that dead hoppers, artificially moved, stimulated marching to the same degree as live hoppers marching nearby, it is possible that hoppers respond to ground vibrations received via the tarsi from other individuals (Chauvin, 1947), when they cannot actually see one another.

The marching activity of isolated hoppers able to see marching individuals nearby was compared with their activity when the nearby hoppers were invisible. The circular, ring-like cage, divided into three concentric galleries was used (Fig. 6B).

Three arrangements were compared, the *controls*, *gallery hoppers* and a new arrangement, *screened hoppers*, in which strips of white cardboard were placed outside the transparent walls of the centre gallery and 20 background hoppers were allowed to march in the inner gallery and 60 in the outer one. A test hopper in the centre gallery could hear and receive ground vibrations from the marching hoppers outside, but was unable to see them.

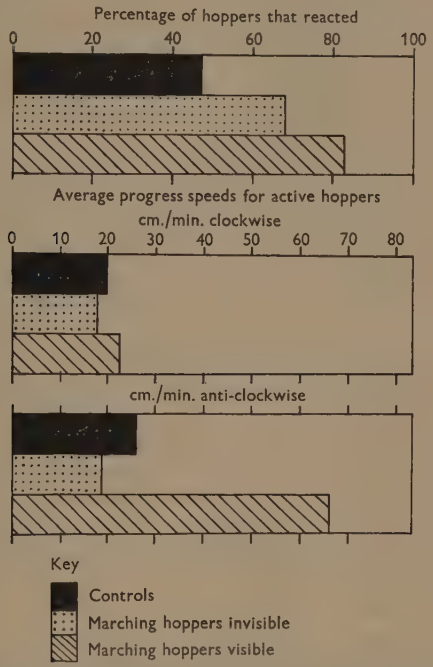

Fig. 9. The marching activity of isolated 4th-instar hoppers able to hear others marching nearby and able to hear and see others marching nearby, in an anti-clockwise direction. Temperature inside the test cage 32° C. (see Fig. 6). Results for 20 females and 3 males. The test hoppers only marched well when able to see others marching, although many were active for short periods when able to hear, but not to see other marching hoppers.

The test hoppers were starved for 1½ hr., tested in one of the arrangements for 10 min., fed for at least 20 min., starved for 1½ hr., re-tested in another arrangement and so on. Each hopper was presented with the three arrangements on one day and six to eight individuals were used in turn. The hoppers were allowed to settle down during the first 5 min. in the apparatus, and during the second 5 min. the divisions of the gallery floor travelled anti-clockwise and clockwise were counted. In 3 days of experiments 23 hoppers were tested.

The results were analysed in three ways (Fig. 9). The percentage of hoppers that moved during the observation period was lowest in the *controls*, highest when the

marching hoppers were visible and intermediate between these two with *screened hoppers*. During these tests, the background hoppers in the inner and outer galleries marched in an anti-clockwise direction. The *progress speeds* were averaged for the ACTIVE hoppers only; speeds in the clockwise direction were similar for the three arrangements, whilst those in the anti-clockwise direction were far higher with *gallery hoppers* than with the other two arrangements. In the *screened hopper* arrangement the test hoppers spent a great deal of the time scrambling up the wall of the inner gallery as if they were trying to go towards the hoppers outside.

The proportion of active isolated hoppers was increased in the presence of other hoppers nearby which were invisible, but sustained marching by the isolated hopper depended on the others being visible to it.

DISCUSSION

Under field conditions, marching is characteristic of hopper bands. The laboratory experiments clearly illustrated an increase in marching activity with increasing numbers of hoppers per cage. Although isolated hoppers marched, averages reached only 32% after 5 hr. of starvation and behaviour varied enormously from individual to individual. This is to be compared with hopper groups of 30 or more, which marched for over 60% of the time after 3 hr. of starvation. The physical conditions which favour marching are not extreme (Kennedy, 1939, 1951; Ellis, 1951) and appear to provide conditions under which marching can express itself. The influence of other hoppers, however, is so great that this factor might well be called a stimulator of marching: a similar conclusion was reached by Clark (1949). As hoppers have a profound effect on each other's activity, those factors which bring and hold the members of a band together are of particular importance. The present experiments illustrated a tendency for hoppers to keep together, but also a tendency for them to spread over the space available, so that marching depended on both cage size and the number of hoppers present. It is not easy to apply these results to field conditions, where there must be a balance between factors causing the disruption and those causing the cohesion of bands. This problem has not been studied in detail, but disrupting forces include heavy rain, cloudy weather and dense vegetation (Kennedy, 1939; Clark, 1949), whilst factors favouring band cohesion include the attraction of hoppers to the same physical environment (Kennedy, 1939), the tendency for hoppers to move parallel with one another (Table 3 and Kennedy, 1951) and a positive attraction of individual hoppers towards groups of their fellows (Clark, 1949).

The experiments on hopper numbers and density illustrated the dependence of marching on both internal and external conditions and the way in which they balanced each other. In the past, a variety of conditions have been shown to play a part in the full expression of marching; e.g. the moulting cycle, the degree of crowding during rearing, the period of starvation, temperature, radiant heat, air currents and other hoppers (Ellis, 1951). In the present tests the two varying factors were the length of starvation and the number of other hoppers. Activity increases with a decrease in the amount of food in the gut (Ellis, 1951) and the

amount of stimulation provided by other hoppers presumably depends on the number of contacts per unit of time, which in its turn varies with the number of hoppers in the cage. Reference to Fig. 3 shows that the time taken for 30% of the hoppers to be marching varied from 5 hr. of starvation with only two hoppers per cage to $1\frac{1}{2}$ hr. of starvation with 100 hoppers per cage. For the same amount of marching, a hopper in a small group (low external stimulation) had to reach a higher level of general activity (i.e. the threshold for sensory stimulation was lowered by starvation for a longer period) than one in a large group (high external stimulation). Presumably, in the field, any one of the factors listed above may limit marching, depending upon the conditions at the time.

The analysis of the fully developed marching behaviour pattern illustrated the importance of optical interactions in increasing hopper activity and supported the theory put forward by Uvarov (1928). Locust hoppers, in common with many other insects, carry out compensating movements when the background is moved (Loeb, 1918). Hoppers appear to experience 'discomfort' when images move across the eye in an anterior-posterior direction, or rapidly in a posterior-anterior direction. The resulting compensating movements cause the hoppers to move in the same direction as the background (Kennedy, 1951); Table 3 illustrates this tendency.

In the laboratory, a moving background increased the proportion of active hoppers and the distance travelled per minute: the *progress speed*. Vertical stripes and live hoppers marching produced the same proportion of reactions, but the *progress speeds* of the reacting hoppers were higher with moving hopper bodies than with vertical stripes. Hoppers began to react to the stripes, but the movement was not sustained. Live hoppers marching and artificially moved dead hoppers gave similar results, suggesting that the jerky movement characteristic of marching hoppers did not play an important part in the opto-motor reaction. The features of hopper appearance (such as shape, size and colour) which bring about the maximal *progress speeds* will be discussed in a later paper: hopper bodies may be more effective than vertical stripes only because their longer outline stimulates a greater number of adjacent ommatidia at any one time (Zerrahn, 1934).

Mechanical interactions between hoppers were necessary for the maximal amount of marching by the maximal number of hoppers in a band. Mechanical interactions increased the proportion of active individuals very considerably, but did not increase the progress speeds of the active hoppers above those obtained with optical stimuli alone. This may indicate that the opto-motor reaction elicits the maximal amount of marching in some individuals, so that further stimulation has no apparent effect: mechanical interactions would then elicit responses in other individuals which will not react to optical stimuli alone. However, mechanical interactions not only caused resting hoppers to move, but they also slowed down active individuals in one of two ways. First, marching hoppers frequently collide; this is always followed by a few seconds pause by the hoppers concerned. Secondly, if a marching hopper comes upon a resting one, it frequently pauses to examine the resting individual. The few blinded hoppers that marched suggest that, on rare

occasions, mechanical interactions alone may produce sustained marching; but most of the experimental results showed that mechanical interactions raised the general activity of the hoppers, while sustained movement in one direction depended on an opto-motor reaction to a moving background.

The other interactions that were tested did not play an important part in marching. Air and ground vibrations from marching hoppers tended to increase the general activity of isolated individuals not subjected to a moving background and may possibly play a part in the movement of bands at night, or under conditions where the hoppers are momentarily obscured from one another.

The laboratory experiments were designed to test increases in marching activity due to optical and mechanical reactions between hoppers. Kennedy (1939, 1951) suggested that such reactions would also keep bands together. It is difficult to agree with this view without appropriate laboratory tests having been made. An examination of Table 3 shows that some hoppers moved against the general stream. In the field, such behaviour by individuals at the edges of the band would lead to a slow dispersal of the hoppers during marching. There are no field observations on this point, although it seems to be assumed that bands do not disperse during marching. If this is the case, then it is reasonable to look for specific reactions that keep bands together, such as a movement towards, instead of parallel with, hopper bodies considerably smaller (farther away) than the hopper itself.

This study has interesting connexions with the feeding of hoppers in the field. Dense populations of locusts could live either fairly evenly dispersed over a wide area, or concentrated into bands so that their distribution is discontinuous. If they are to avoid starvation, dense populations of hoppers must move about actively. Concentration into bands has the advantage that it increases hopper activity and the distance travelled per day. Hoppers of *Locusta migratoria* march under normal conditions; but if food becomes short, then after a few hours of starvation very vigorous marching takes place, which should increase the chances of the hoppers coming upon a new food supply. It seems that starvation does not normally play an important part in the marching of this species, but in *Chortoicetes terminifera* (which in many ways is intermediate between grasshoppers and locusts like *Locusta migratoria*) the hoppers generally march after they have eaten all the food in one area (Clark, 1949). In more typical locust species marching occurs before the food in one area has been eaten, probably because their hopper interactions result in greater activity than those of *Chortoicetes terminifera*. Comparative studies of species which are intermediate between typical locusts and grasshoppers may indicate how the mass movements of locusts have evolved.

SUMMARY

1. The amount of marching by 4th-instar hoppers of *Locusta migratoria migratorioides* (R. & F.) which have been reared in crowds, was studied in relation to the total number of individuals and to the size of the cage.

2. Below 50 hoppers per cage, marching depended partly on the total number of hoppers present and partly on the number in relation to the area of the cage floor.

Above 50 hoppers per cage, marching probably depended on the total number present, rather than the area of the cage floor.

3. In all cages, marching was greatly reduced with fewer than 30 hoppers, although even single individuals marched for part of the time.

4. An investigation of the hopper interactions which lead to an increase in marching activity in groups showed that the two most important were visual and mechanical ones.

5. Test hoppers followed moving hopper bodies alive or dead, but only gave a partial response to moving vertical stripes.

6. Mechanical interactions between hoppers greatly increased the proportion of reacting hoppers, but did not increase the distance travelled by the active individuals, beyond those travelled in response to optical stimuli alone.

7. Maximal marching activity, by the maximal proportion of hoppers in a band, depended on an opto-motor response to a moving background of hopper shapes and to the physical contact between hoppers.

8. Air and floor vibrations from other marching hoppers played only a small part in marching, whilst stimuli received via the antennae tended to inhibit marching when total activity was low.

9. The importance of the experimental results in explaining the behaviour of locust hoppers in the field was discussed.

I have to thank the Anti-locust Research Centre for grants that enabled me to carry out this research; Prof. D. M. S. Watson and Prof. P. B. Medawar for giving me facilities to work in the Zoology Department of University College, London; Dr B. P. Uvarov and Dr D. L. Gunn for helpful discussions; Mr Hunter-Jones and others at the Anti-locust Centre for the supply of animals and Mr Redpath of University College for making the moving background cage.

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THE INFLUENCE OF DISSOLVED OXYGEN ON THE RESPIRATORY MOVEMENTS OF CADDIS LARVAE

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Caddis larvae which bear cases ventilate their gills by undulatory movements of the abdomen. These respiratory movements produce a stream of water flowing out through the posterior opening of the case. In well-aerated water the movements are intermittent, periods of undulation alternating with pauses. In poorly aerated water the pauses become shorter.

The only study of the environmental control of these respiratory movements by caddis larvae is that of van Dam (1938). This worker studied the larvae of *Phryganea grandis* L. which had been removed from their cases and confined in celluloid tubes open at each end. He found that raising the temperature shortened and finally abolished the pauses between bursts of undulation. The same result was obtained when the oxygen content of the outside water was lowered by bubbling nitrogen through it. When aerated water was passed directly through the celluloid tubes, respiratory movements ceased. Carbon dioxide caused no acceleration in the movements. It appears then that in nature a rise in temperature or a decrease in dissolved oxygen augments the amount of ventilation of the gills. Van Dam observed that the amplitude of the respiratory movements is also thus increased. He saw, too, that crawling decreases the pauses, doubtless through oxygen-lack. He also observed several strong and rapid ventilation movements after defaecation, an adaptation to wash faeces out of the case.

These observations are very interesting, but they are inadequate since no measurements were made of the oxygen content of the water, nor is it stated how many individuals were studied. Moreover, the tubes in which the animals were confined do not imitate conditions in nature. We therefore thought it worth while to make a further study of the question. We used the larvae of *Limnophilus flavicornis* Fabricius, from the Long Water at Hampton Court. Two days before an experiment, animals were removed from their cases and given pieces of cellophane of about the same size as the material used in nature for case-building. With this substance the larvae constructed transparent cases. For observation the animals were placed in stoppered glass bottles of water, immersed in a glass tank of water at 20° C. The respiratory movements of each animal during a period of 8 min. were recorded on a smoked drum with a lever actuated electrically by a tapping key. This gave the time of each movement, but unfortunately not the amplitude. The number of beats per minute was later computed.

In each of 25 experiments on the influence of oxygen, a record was first made of the respiratory movements in fully aerated water, and then this water was siphoned off from the bottle and replaced by water of a low oxygen content, previously prepared by bubbling nitrogen through it. After a second record of the

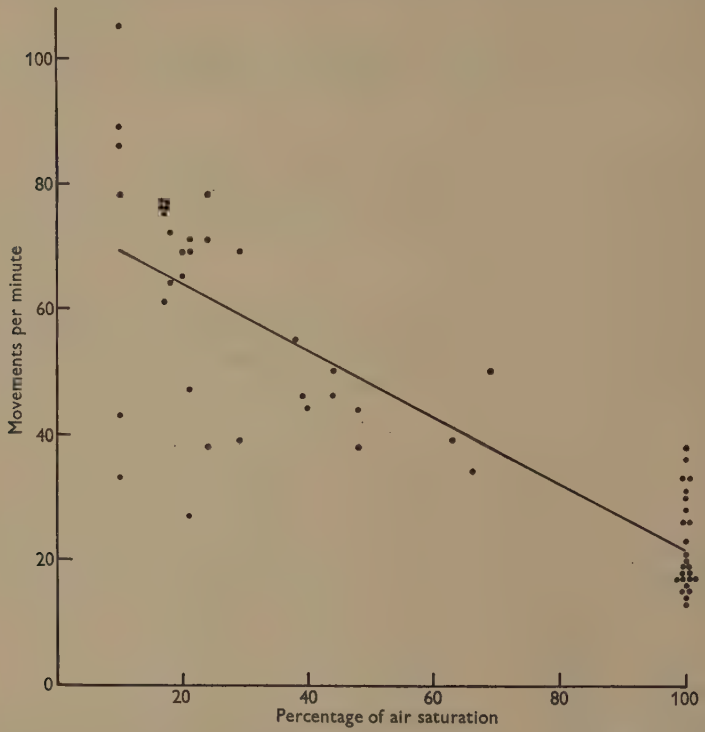

Fig. 1. The relation between the oxygen content of the water and the rate of respiratory movements in the larva of *Limnophilus flavicornis*. Each of the 60 points is a mean rate of movement calculated from a record of the number of movements in a larva during 8 min. at 20° C.

movements, the stopper was again removed and a sample of the water was analysed for dissolved oxygen by the micro-Winkler technique of Fox & Wingfield (1938).* In ten of the experiments a third record was made at a lower oxygen content. We found irregular pauses in the ventilation by the larvae in air-saturated water, and we confirmed van Dam's observations of the abolition of pauses and the increased amplitude of movement in poorly aerated water, and also of the special movements after defaecation.

* The analyses were kindly made for us by Miss Barbara M. Gilchrist.

Our results are given in Fig. 1, where the oxygen content of the water is expressed as a percentage of air saturation, which, at 20° C., means an oxygen concentration of 6·4 ml./l. The mean number of movements per minute of each of sixty records is entered in the figure. It is clear from the figure that a low oxygen content of the water increases the rate of respiratory movements. The correlation coefficient between percentage air saturation (x) and movements per minute (y) is $r = -0{\cdot}841$ ($P < 0{\cdot}001$). (A less high correlation coefficient is obtained between percentage air saturation and the logarithms of movements per minute.) The regression line of y on x is given by $y = 74{\cdot}61 - 0{\cdot}527x$, and has been inserted in Fig. 1.

We have confirmed the observation of van Dam that carbon dioxide does not accelerate the respiratory movements. The experiments were made in fully aerated water to which small quantities of water saturated with carbon dioxide had been added to reduce the pH to definite measured values. In thirty-three records on the drum of respiratory movements at 20° C., the mean rate of movements, namely 20 per minute, was the same at pH 8·0 and 7·5, whilst at pH 6·7 the rate fell to 13 and at pH 6·4 to zero. The effect of carbonic acid at the low pH values was narcotic, for the normal rate was resumed on return to aerated water.

SUMMARY

A quantitative study has been made of the effects of dissolved oxygen and carbon dioxide on the respiratory movements of the larva of *Limnophilus flavicornis*. A diminution in oxygen accelerates the movements but an increase in carbon dioxide has no such effect.

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RELATION OF THE RATE OF ANTENNAL MOVEMENT IN *DAPHNIA* TO THE NUMBER OF EGGS CARRIED IN THE BROOD POUCH

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The number of parthenogenetic eggs and embryos carried in the brood pouch of *Daphnia* varies greatly. In some natural populations of the pond-dwelling species females carry many eggs,* in other populations they have few. We have found in nature a female of *D. magna* Straus carrying 95 eggs, and de Kerhervé (1926) had one in culture with 105 eggs, whilst, on the other hand, in some natural populations of the same species no more than one, two or three eggs are borne. Two potent causal factors influencing egg number are the quantity of food and the aeration of the water: up to a certain maximum level of feeding, the number of eggs produced is proportional to the quantity of food eaten (Ingle, Wood & Banta, 1937; Fox, Hardcastle & Dresel, 1949), and below a certain degree of aeration of the water the egg number falls off proportionately to the amount of dissolved oxygen (Fox, Gilchrist & Phear, 1951). In addition, fewer eggs are produced at a high temperature (MacArthur & Baillie, 1929; Fox & Phear, 1953). Other factors influencing the egg number are the nature of the food (Lefèvre, 1942) and the age of the mother (Anderson, Lumer & Zupancic, 1937).

Daphnia swims by oar-like movements of its pair of antennae. Swimming is necessary both for progression and to keep the body from sinking. The eggs are heavier than water, since isolated eggs sink in water; therefore the specific gravity of an animal with many eggs may be greater than that of one with few eggs in the brood pouch. This might be expected to necessitate swimming more vigorously in order to keep up in the water, and the rate of antennal movement would then increase with the number of eggs. Another factor intervening is the dorsal and posterior position of the brood pouch containing the eggs, which must change the centre of gravity of the female and perhaps necessitate a modified mode of swimming. With all this in view, we set out to count the rate of antennal movement of parthenogenetic females of *D. magna* carrying different numbers of eggs.

The time to 0·1 sec. for ten antennal strokes was taken with a stop-watch. This was done 10 times for each animal and the mean reckoned, from which the rate of antennal movement, in beats per minute, was calculated. Each animal was studied singly in tap water at 20° C., with standard illumination. Subsequently, the number

* In this paper 'eggs' in the brood pouch means 'eggs or embryos'.

of eggs or young was counted, after removal from the brood pouch if numerous. Of 103 individuals studied, sixty-five bore eggs and thirty-eight had empty brood pouches.

The results are shown in Fig. 1, where each point gives the relation between the mean rate of antennal movement and the egg number for one animal. It is clear

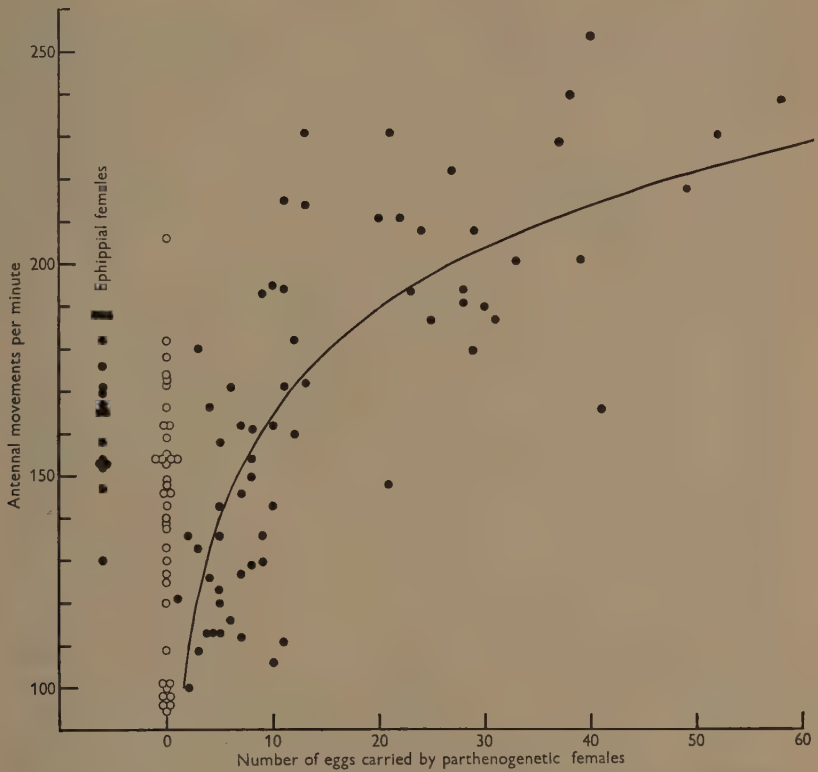

Fig. 1. Relation of the rate of antennal swimming movement of *D. magna* to the number of eggs or young carried in the brood pouch, or to the presence of an ephippium. Each point refers to one individual.

that the rate of antennal beat increases with the number of eggs carried in the brood pouch. It is also evident that the relation between the two variables is not a linear one; indeed it could not have been such, or the rate of beat for 100 eggs would have been impossibly fast.

Statistical analysis of the relation between rate of antennal movement (v) and egg number (n) (excluding animals with no eggs) shows that a higher value for the correlation coefficient is obtained when it is assumed that v is proportional, not

to n, but to $\log_{10} n$. On this assumption the correlation coefficient has the value of 0·79 ($P < 0{\cdot}001$) and the regression equation is

$$v = 83{\cdot}4 + 81{\cdot}7 \log n.$$

For this regression a second-order polynomial equation is

$$v = 91{\cdot}8 + 8{\cdot}6n - 0{\cdot}12n^2.$$

The curve of this equation has been drawn in Fig. 1.

The mean rate of antennal movement of each of the thirty-eight animals carrying *no eggs* in the brood pouch is also entered in Fig. 1. It is clear that the values have a wide scatter. For these animals the mean rate of antennal movement to be expected from the regression equation is of the order of 75 beats per minute, with a standard error of 4·6. Less than one half of the thirty-eight values fall within the upper 95% confidence limits for the regression equation, and it must therefore be presumed that many of the animals carrying no eggs belong to a different statistical population from those with eggs.

Counts were made, too, of the rate of antennal strokes of eighteen females with *ephippia*. These data are also entered in Fig. 1. It is seen that the rate is midway between the fastest and slowest rates of parthenogenetic egg carriers.

It was necessary next to find if a possible greater specific gravity of animals with many eggs does make them sink more rapidly. This was tested by timing the fall, through a certain distance in water, of dead *D. magna* carrying varying numbers of eggs. It was done in a 1 l. glass measuring cylinder containing tap water at 20° C., within a glass museum jar of water acting as a thermostat. Rubber bands marked two levels 30 cm. apart in the cylinder, with 10 cm. of water above the upper band. Each individual *Daphnia* was killed by momentary immersion in water at 50° C. and then pipetted gently into the cylinder. Thirty-three individuals with and without eggs were tested but no significant difference was found between the rates of fall with many, few or no eggs.* This uniform rate of fall must mean that friction with the water has a greater effect on sinking rate than small differences in specific gravity due perhaps to different numbers of eggs. In animals killed by heat the antennae were spread out†, and, with their setae, they must have acted as parachutes. In life the antennae are, of course, not always spread out, but only during part of each swimming stroke. Between strokes heavier animals may sink faster.

A study was also made of a possible influence of food contained in the gut on the sinking rate of *D. magna*, as different amounts of gut contents might have accounted for some of the scatter of points in Fig. 1. Eight individuals were kept for 2 days without food in several changes of tap water so that their guts were more or less emptied. The rate of fall of these animals through 30 cm. of water, after killing them as before, was compared with that of eight freshly caught individuals with full guts. But the gut contents showed no effect on rate of fall. Eyden (1923) had tested this by keeping four individual *D. pulex* for 8 days, feeding them in the day-

* Eyden (1923) concluded that narcotized *D. pulex* sinks more slowly after the young leave the brood pouch, but she studied only one individual!

† Narcosis with urethane was tried, but was abandoned because the position of the antennae was not uniform, as it is in animals killed by heat.

time and starving them at night. She narcotized them every morning and evening, and timed their rate of fall through 20 cm. of water. She found that they fell quicker in the evening.

It was suggested to us by Dr Barbara M. Walshe that since egg number in *Daphnia* depends upon feeding, individuals with many eggs might move their antennae more quickly merely because they were well fed, just as a horse is 'fresh', or fit for hunting, after being given extra corn. To test this in the absence of eggs we had recourse to males of *D. magna*. They were fed for 5 days on *Chlorella*, one lot receiving 10 times as much food as the other. Counts of the antennal rates of fifteen individuals of each lot showed no significant difference in the means, so this hypothesis was abandoned.

We conclude that the rate of swimming movements in *Daphnia* increases with the number of eggs or embryos in the brood pouch. This is not a direct result of abundant food, which might have resulted in greater swimming vigour. Although eggs may augment the specific gravity of *Daphnia*, yet dead animals with many eggs do not sink faster than those with few eggs or none. The faster rate of swimming movements of those carrying more eggs may, however, arise from the position of the brood pouch, which is like a knapsack, changing the animal's centre of gravity in proportion to the number of eggs it contains. The mode of swimming of *Daphnia* has been described by Scourfield (1900). Between each antennal stroke and the next one the animal sinks with its tail-spine leading. The antennae beat backwards, dorsally to the line from head to tail-spine, thus making the head incline forwards. A knapsack of eggs must prevent some of the forward tilt. Therefore, to swim at the same water-level as an eggless female, one carrying eggs should make more frequent antennal strokes.

Individuals of *Daphnia* carrying many eggs are, on the whole, bigger than those with few eggs. This is to be expected, since abundant food increases both body size and egg number (Ingle, *et al.* 1937). We measured the length of 114 egg-carrying individuals of *D. magna* with an eyepiece micrometer, taking the distance from the crown of the head to the base of the tail-spine: the biggest measured 4·9 mm. and the smallest 2·3 mm. In each of these individuals we counted the number of eggs in the brood pouch, which varied from 1 to 58. The correlation coefficient between length and egg number was found to be $+0{\cdot}55$ ($P<0{\cdot}001$).* Thus the rate of antennal movement, which increases with the number of eggs carried, necessarily increases also with the size of the female. Could it be, then, that the correlation found between rate of antennal movement and number of eggs is really one between rate of antennal movement and size of female? This is not so, for the following reason. We made measurements of the lengths of thirty-three of those females carrying no eggs whose antennal rates are shown in Fig. 1: the extreme values were 4·2 and 2·3 mm. There was no connexion here between size of animal and rate of antennal movement, for the correlation coefficient was only 0·06. Thus antennal rate is proportional to size of clutch, but merely incidentally to size of animal.

* Big animals with numerous eggs did not have smaller eggs; measurements were made to test this possibility, but no evidence was found in its favour.

SUMMARY

The rate of antennal swimming movements of *Daphnia* is proportional to the number of eggs or young carried in the brood pouch. An increased rate of antennal movement is not a direct result of the better nutrition which produces more eggs. The increased swimming rate may be necessary to counteract a change in centre of gravity caused by the posterior position of the brood pouch containing eggs.

We wish to thank Dr R. J. Whitney for statistical help.

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SUN NAVIGATION IN HOMING PIGEONS

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INTRODUCTION

In an earlier paper (Matthews, 1951*b*) it was definitely established that homing pigeons could truly *navigate*. That is, when released in an unknown area in a novel direction they showed a strong initial homeward orientation, and a good proportion returned home in times that would admit of little deviation from the line of direct flight. This has been confirmed by Kramer & St Paul (1952) and by Kramer (1953). Similar evidence of navigation has been obtained with the migratory Manx Shearwater (Matthews, 1952*b*, 1953*b*) and, as regards initial orientation, with the Lesser Black Back Gull (Matthews, 1952*a*).

/The experimental work with pigeons had eliminated the possibility of navigation by use of the earth's magnetic field or of an acceleration/displacement recording mechanism./But with all three species indications of a breakdown of the orientation with overcast skies had been obtained. The present paper reviews the evidence that has accumulated for the homing pigeon on this effect, and describes experiments aimed at an analysis of the proposed sun-navigation hypothesis.

MATERIAL AND METHODS

The general technique employed was described in detail in the earlier paper (Matthews, 1951*b*). Young pigeons, 3–5 months old, were used. In 1948–50 they were operated from the lofts of several local pigeon experts, in 1951–2 from a loft established at the Ornithological Field Station, Madingley, Cambridge. They were 'trained', starting in July, by releases in small groups at gradually increasing distances in one direction up to 10–12 miles. Then followed releases at 25, 25, 50, 50, 80, 80 and 130 miles on the same 'training-line', with the birds released singly (watched out of sight with 16×40 binoculars before the next was released). Critical releases were made in late August and September off the 'training-line'. The experimental releases at 25 miles or more are numbered for reference: 1948, A and B; 1949, 1–9; 1950, 10–21; 1951, 22–28; 1952, 29–36; with the prefix 'T'. They involved a total of 233 birds in 1306 sorties, for which three indices of performance are available:

Vanishing point	Bearing at which bird was lost from sight.
Orientation time	From release to vanishing.
Homing success	Time to reach home.

The interrelation and value of these indices has been discussed earlier. By far the most useful for throwing light on the orientation process has been the first, represented in radial scatter diagrams, e.g. Fig. 1. Their statistical significance can be tested by considering the mean angular deviation from the home direction, regardless of sign—since, in general, a deviation to the right is as good, or as bad, as an equal deviation to the left. The mean deviation ($\bar{x}$) could then be subjected to a 't' test to see whether it differed significantly from a random distribution, which will have a mean deviation ($\bar{x}_r$) of 90°. In practice it is more realistic to make the comparison with a regular radial scatter of approximately the same number (N_r) of observations. This will still have a mean deviation of 90° but, unlike the ideal random scatter, an error introduced by the essential discontinuity. From the resultant value of t_r* the probability (P) of the scatter under test occurring by chance can be estimated. If $P > 0{\cdot}05$ the scatter cannot be differentiated from random.

A check on the regularity of the distribution is given by a χ^2 test. If this should indicate non-randomness, whereas the 't' test indicated randomness, the conflicting result may have two causes. The distribution may be askew, and this can be tested by recalculating t_r about the algebraic mean (i.e. taking sign into account). If this fails to resolve the conflict, a second possibility is that the distribution should really be considered as being, for instance, bimodal, with concentrations about two, particularly opposite, points. The boundaries of the distributions have to be determined by inspection. These refinements are only of importance when the birds have a conflicting choice of orientation lines, as when the home direction differs markedly from that in which they have been trained. For some purposes it is more appropriate to compare the proportion of points falling within a particular sector, and here the χ^2 test is applied.

For comparing orientation times and homing successes, straightforward 't' or χ^2 tests are used as appropriate. An attempt has been made to save the general reader the boredom of constant repetition of the phrases 'this is significant' and 'this is not significant'. As far as possible the appropriate word or words of a comparison are *italicized* when tests have shown them to have statistical significance—thus, *random*, *different*, *higher*, *less*, etc.

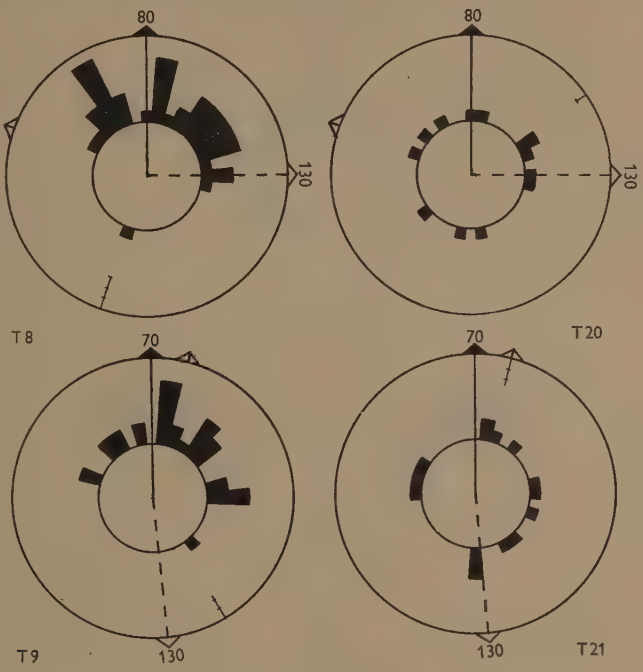
OBSERVATIONAL EVIDENCE FOR A FORM OF SUN NAVIGATION

We may consider first the initial orientation patterns that have resulted when pigeons are released: (*a*) in sunny conditions, with well-broken cloud, 5/10th or less, (*b*) with complete cloud cover of such a thickness that the sun cannot shine through. When two lots of birds are released at the same unknown point, either

$$* \; t_r = \frac{(\bar{x} \sim \bar{x}_r)\sqrt{\left[\frac{NN_r(N+N_r-2)}{N+N_r}\right]}}{\sqrt{\left[S(x^2)-\frac{[S(x)]^2}{N}+S(x_r^2)-\frac{[S(x_r)]^2}{N_r}\right]}}.$$

80 miles WSW. or 70 SSE. after previous training to 130 miles NNW., we have the result shown in Fig. 1.*

χ^2 tests confirm that the distribution with overcast is *random* in both cases, and *strongly* orientated in the home direction with sun. These are the conditions that would particularly call for accurate bico-ordinate navigation. With releases in the training direction, bico-ordinate navigation may still function, but if it becomes

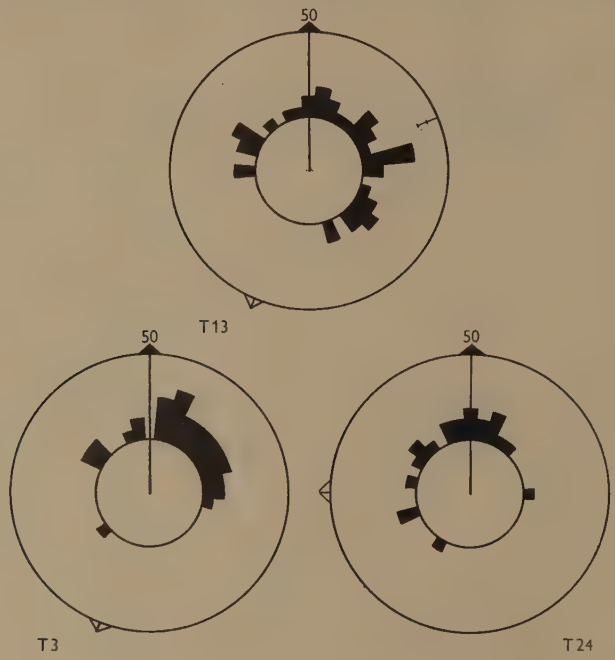


	Sun		Overcast	
	T8	T9	T20	T21
N	47	31	13	16
$\bar{x}$	45°	45°	78°	93°
σ	30°	33°	54°	59°
P	<0·001	<0·001	0·6	0·8

Fig. 1. Effect of cloud conditions on initial orientation at right angles (top) and in opposite direction (bottom) to training line.

* Explanation of Fig. 1 and other radial scatter diagrams. In all cases, except Fig. 4, the results shown are those of birds released at the point for the first time. The vanishing points are grouped into 10° sectors. Solid arrow—home direction and distance. Dotted arrow—training direction and distance. Shaftless arrow-head—true north. Thin, centripetal line—wind direction and Beaufort Force, indicated by cross lines. *N*, number of pigeons; $\bar{x}$, mean angular deviation regardless of sign; σ, standard deviation; *P*, probability, based on a '*t*' test, that such a scatter could have arisen by chance.

difficult it may be replaced by simpler navigation in one co-ordinate, i.e. if the overcast allowed a very rough estimate of the sun's position this would be sufficient for a homeward tendency to result merely from the birds flying in the training direction. Therefore the effect of overcast would not be expected to be so clear-cut. Nevertheless, at the early stage of 50 miles on the training line, the results in Fig. 2 were obtained. Again there is *good* orientation in sunlight, *random* scatter with

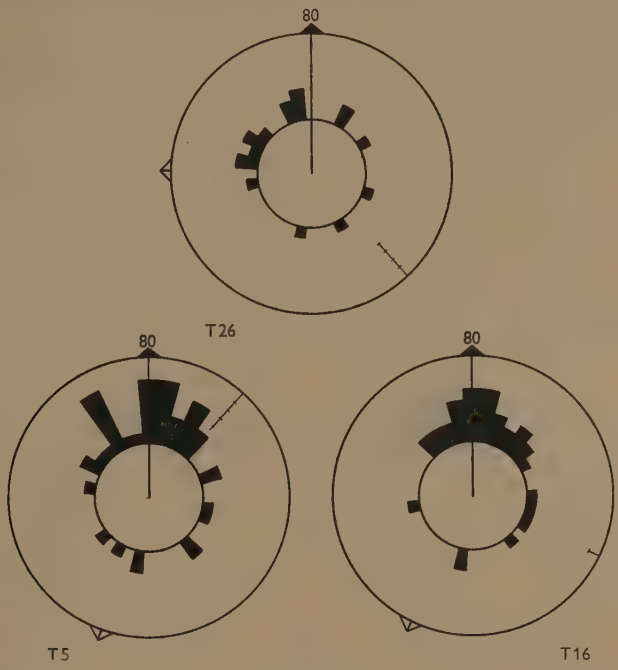


	Sun	Overcast	Sun
N	37	42	23
$\bar{x}$	46°	78°	41°
σ	30°	45°	41°
P	<0·001	0·3	0·004

Fig. 2. Effect of cloud conditions on initial orientation on training line at 50 miles.

overcast (as there is no conflict of directions, the right-handed bias in T 13 does not discredit the verdict of the 't' test). At the following, 80-mile stage, where the effect of repeatedly flying in the same direction would be expected to be more marked, the same general result is obtained (Fig. 3). The overcast release was again *random*, though closer to the significance level than in the previous cases. Comparison may also be made between the two stages. Where there is overcast in the first and sun in the second (T 13 *v.* T 16) a *big* improvement in orientation is seen

($P < 0{\cdot}001$). Where there is sun on both occasions (T3 *v.* T5) there is a slighter improvement. But when an overcast release follows a sunny one (T24 *v.* T26), the second is worse than the first—the effect of overcast overrides the improvement expected after greater experience and the weeding out of some unsuccessful birds. This is even better shown in cases where a second release at the same place was made with overcast. Fig. 4 illustrates this point. In both cases the primary release



	Sun	Overcast	Sun
N	46	18	39
$\bar{x}$	47°	62°	47°
σ	48°	47°	42°
P	$<0{\cdot}001$	$0{\cdot}1$	$<0{\cdot}001$

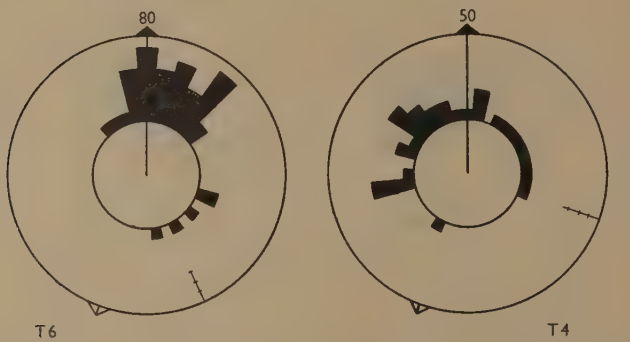
Fig. 3. Effect of cloud conditions on initial orientation on training line at 80 miles.

was made in sunny conditions. The repeat T6 showed an improvement over the previous T5, while the overcast repeat T4 was worse than the previous T3. It has been shown by laboratory experiments (Matthews, 1952*c*) that the learning of landmarks probably does not play an important part in the orientation and homing of pigeons, the present comparison confirms that this is the case, i.e. that it is much more important that the birds should have a good view of the sun, than that they should have had previous experience of the release point.

The second index of performance, orientation time, is particularly liable to influence by irrelevant factors, such as birds waiting around for another bird to be released, and by visibility conditions. Nevertheless, comparing the data for sun and overcast releases we have:

Minutes	Off training line				Training line at 50 miles		
	Sun		Overcast		Sun, T3	Overcast, T13	Sun, T24
	T8	T9	T20	T21			
$\bar{x}$	3·3	4·4	7·8	7·5	5·0	4·2	3·7
σ	1·1	2·3	3·1	7·1	3·0	2·0	2·4

Minutes	Training line at 80 miles			Repeat releases	
	Sun, T5	Overcast, T26	Sun, T16	Sun, T6	Overcast, T4
$\bar{x}$	4·7	5·6	2·7	4·7	5·6
σ	1·9	2·5	1·5	2·4	3·2



(cf. T5, Fig. 3) (cf. T3, Fig. 2)

Sun		Overcast
42	N	34
35°	$\bar{x}$	57°
40°	σ	36°
$<0{\cdot}001$	P	0·005

Fig. 4. Effect of cloud conditions on initial orientation for second time at a point.

Thus in six out of seven cases the overcast orientation time was greater than the equivalent sun value. The exception, T3 *v.* T13, would seem to be due to an abnormally high value for the former. The birds concerned in T13 showed a marked drop in orientation time when released at the next stage, T16, in sunny conditions, whereas those in T3 showed only a slight reduction. The differences are *significant* in the two releases off the training line ($P<0{\cdot}001$ and 0·04), and

between T16 and T26 ($P<0.001$). If we amalgamate the remainder we have:

	Sun	Overcast
N	148	94
$\bar{x}$	4·6	5·0
σ	2·5	2·0

The difference, although small, is *significant* ($P<0.001$). We can therefore conclude that with overcast there will be considerably more lingering near the release point than in the equivalent sunny conditions. Indeed, since the former produced *random* scatters the relation between orientation time and accuracy of orientation noted before (Matthews, 1951*b*) should not apply. And this is indeed the case. Comparing the proportions falling within the 45° arc centred on the home line we have:

Orientation time (min.)	Sun		Overcast	
	N	Within 45°	N	Within 45°
Less than 3·5	146	41%	38	21%
More than 3·5	119	27%	85	19%

Under sunny conditions birds quickly lost from sight gave a *higher* ($P=0.02$) proportion of vanishing points close to the home line. With overcast there is no difference between the two categories.

The third index of performance, homing success, is the least likely to show a relation to conditions at release. These are unlikely to be maintained over the whole homeward track. Chances of a badly orientated bird reaching home are quite high at the shorter distances. Speeds of return are considerably affected by the wind force and direction, varying from one test to another, or on the day of release. Detailed examination of individual orientation and homing histories (Matthews, 1952*c*) shows that while there is a general positive relation, marked exceptions are fairly frequent. Bearing these important qualifications in mind, and considering only the proportion of birds back on the day of release and their average speeds, we have:

	Off training line				Training line at 50 miles		
	Sun		Overcast		Sun, T3	Overcast, T13	Sun, T24
	T8	T9	T20	T21			
Back on same day (%)	53	71	38	44	89	81	61
Speed (m.p.h.) $\bar{x}$	23	18	11	12	18	21	11
σ	10	8	3	2	8	7	7

	Training line at 80 miles			Repeat releases	
	Sun, T5	Overcast, T26	Sun, T16	Sun, T6	Overcast, T4
Back on same day (%)	87	22	72	98	87
Speed (m.p.h.) $\bar{x}$	20	15	22	31	17
σ	6	8	9	9	4

At the shorter distance on the training line, conditions at release do not affect homing success. In all the other cases sunny conditions gave more and faster returns on the day than did overcast. The differences are only significant for the proportion returning at the 80-mile training-line point ($P < 0{\cdot}001$), and for the speed in the repeat releases ($P < 0{\cdot}001$).

Kramer (1953) has also found, on a small scale, that overcast disorientates pigeons.

To summarize this observational data, we have seen that with sunny conditions good homeward orientation is obtained. With overcast the scatter from the release point is random, and more time is spent near it. The conditions at release also have a discernible effect on homing success. For pigeons, therefore, there can be no doubt that the sun plays an essential part in their navigation. When we add the very similar results obtained with gulls (Matthews, 1952*a*) and shearwaters (Matthews, 1952*b*, 1953*b*) the case for a form of sun navigation is strong indeed.

THE HYPOTHESIS

It is strange that so obvious a means of navigation, and one used by ourselves, should only have recently been considered as applicable to bird orientation. The theories of random/systematic search, of navigation by means of the earth's magnetic field and of displacement/acceleration measurement all had their beginnings around 1870. But it was not until 1945 that Ising, as an adjunct to his Coriolis theory, suggested that birds might determine longitude displacement by time differences in sunrise or sunset. He went further in recognizing that if latitude displacements were large only the noon position of the sun would be suitable for longitude estimation. But he overlooked the fact that latitude could be measured from the sun's altitude at that point. Varian (1948), Davis (1948) and Wilkinson (1949) independently suggested that this could be done, but overlooked the possibility of longitude determination from the sun. Matthews (1951*a*, *b*) put forward a hypothesis for complete sun navigation, deriving both latitude and longitude from the sun's position alone. As the essential part played by the sun has since been more clearly demonstrated, it is appropriate to re-state and re-examine this hypothesis here. The suggestion is:

(1) By observation of the sun's movement over a small part of its arc, and by extrapolation, the highest point of the arc is determined. This gives geographical south and local noon.

(2) Comparison of the remembered noon altitude at home with the observed noon altitude gives the difference in latitude.

(3) Comparison with home position in azimuth at local noon gives the difference in longitude. Alternatively, this might be appreciated as a direct time difference.

(4) All measurements and comparisons are automatic.

Fig. 5 will make these conceptions clearer. Ptolemaic terms are used for simplicity, i.e. the sun moves and not the earth. The formula is the simplest one that will give both latitude and longitude from the sun's position alone. The requirements and assumptions implicit in it may now be considered in detail.

If there were some means of determining the south reference point independently, the first item of the hypothesis could be omitted, and the sun's position as observed compared directly with its position at home *at the same instant*. But any possibilities of such an independent compass have been eliminated. A magnetic one is discredited by the experiments of Gordon (1948), Matthews (1951*b*), Yeagley (1951) and van Riper & Kalmbach (1952). In any case it would still require a correction for local declinations, which would necessitate a knowledge of the very fact

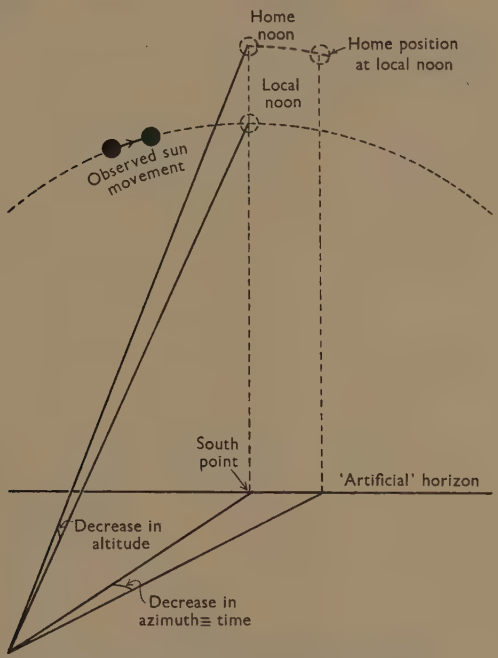


Fig. 5. Diagram illustrating the hypothesis of sun navigation. Release to north and west of home. (Not to scale.)

required, geographical south. A gyro-compass, requiring a rapidly rotating gyroscope, is a morphological impossibility. A bearing on the Pole star would give the reference point, but to see stars in daylight would require a development of the means used by astronomers to view faint stars—observation in optical blackness through a very small opening—described by Pirenne (1948). Not only is the pigeon's eye structurally unsuited for this, but such a method would make the localization of one star—usually by reference to constellation patterns—almost impossible. It is relevant to note that for conditions in which the magnetic compass is unreliable, near the Poles, a semi-automatic *sun*-compass has been developed for aircraft navigation (Wright, 1950). Kramer (1953) attempted to confuse pigeons by releasing them at the exact instant when the sun at the release point

was at the same height as it was at home. No confusion resulted, as would be expected on the present hypothesis. The avian eye appears well adapted to movement detection, in which the pecten may play an important part (Menner, 1938; Crozier & Wolf, 1943; Griffin, 1952). When we ourselves have some fixed point to observe close to the sun, the latter's movement becomes very obvious, and the blind spots produced by the pecten may function in an analogous way.

Good orientation has been obtained when the horizon was hilly or indistinct through haze. It is therefore necessary to postulate that birds have the equivalent of the bubble sextants (Bennett, 1941) or artificial horizons that enable airmen to measure altitude without reference to the visible horizon. Certainly the band-like *area*, with pigmented borders, found in many birds, though not in pigeons, looks through an ophthalmoscope (Wood, 1917) temptingly like the base-line of an aircraft's artificial horizon. Griffin (1952) has suggested the pecten for this role. Certainly there is good reason to think that the bird's head has the necessary stability in flight to serve as an 'instrument-bed'. Dr R. J. H. Brown has taken a long series of electronic flash cine-photographs of pigeons in fast flight (Brown, 1953), and very kindly permitted me to examine his original prints. The pigeons flew at speeds of from 13 to 26 m.p.h. down a corridor past a squared backcloth. Within the limits of measurement (scale approx. $\frac{1}{10}$th) there is no trace of an up/down movement of the eye's centre in relation to the wing beat cycle. Any changes in the vertical plane were steadily up *or* down, and were very small. For twelve sequences the ratio of horizontal movement to vertical movement averaged 107 : 1 (S.E. 27). The best case was that of a bird flying at 16 m.p.h. through a complete beat cycle (up/down/up) and covering approximately 99 cm. forward with the eye centre moving down less than 0·3 cm.—a ratio of 330 : 1. If such steadiness is achieved at a relatively low speed, in enclosed surroundings, it is not improbable that even better results would be achieved in normal conditions.

The sun's arc rises and falls across the sky with the changing seasons, reaching a maximum altitude on 22 June and a minimum on 22 December—the two solstices. At these dates the daily rate of change in altitude is very small, about 10″ of arc, but it increases to a maximum of about 1400″ of arc at the two equinoxes, 21 March and 23 September. Even if the maximum rate of change (equivalent to 27 miles of latitude) was not allowed for by the bird, no gross error would be introduced in the conditions under which the previous experiments have been made—birds allowed full view of sun and sky on the day before release, taken overnight to points 50–130 miles away and kept covered until release. Nevertheless, this seasonal factor allows an experimental opening which has been exploited (p. 257).

The existence of some form of accurate time mechanism is an essential part of the full hypothesis, though in theory latitude could be obtained without such a chronometer, from the altitude of the highest point of the sun's arc. Until accurate chronometers were available the only method of measuring longitude open to sailors was to start a less accurate clock at local noon (determined by the highest point) and note the time at which some forecast heavenly event occurred. An almanac would give the time after local noon at which the event would occur at,

say, Greenwich, and the difference in times would give a rough idea of longitude. This still requires some form of clock, and cannot be used by pigeons, since it is beyond fantasy to provide them with an almanac, and the only suitable events during the day would be the infrequent eclipses of the sun, and the extremely rare transits of Mercury and Venus.

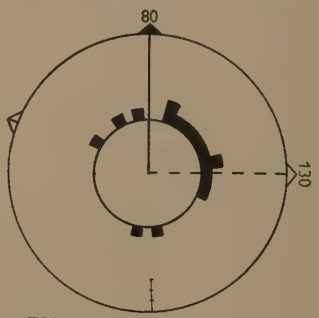
The evidence for some form of general time-keeping mechanism is widespread in animals, as for instance in diurnal rhythms. A more particular time sense has been demonstrated in insects (Beling, 1935, for summary) and in passerine birds (Stein, 1951), in which the animals could be trained to come to food at a particular hour of the day. Stein claims that this time sense was not disturbed by being kept in constant light, by a varying length of fasting before feeding, or by the injection of metabolic drugs. His evidence, particularly of the last two points, is not wholly convincing, and certainly does not permit a decision on whether such a clock would have the necessary accuracy for navigation purposes. The field is clearly wide open for more detailed research. Starlings and pigeons (Kramer 1950; Kramer & Riese, 1952) trained in a particular direction in a laboratory situation can take up the direction with reference to the sun's position in azimuth regardless of the time of day. This confirms work with pigeons in the field by Matthews (1951*b*). So in the present state of evidence, the existence of some form of chronometer is very likely, though almost nothing is known as to its physical basis or its limits of accuracy.

The present hypothesis differs from other 'super' sensitivity theories in two important ways. First, if the displacement is large and swift, say of the order of 500 miles, one could be confident of obtaining a rough fix oneself by the method proposed, without instruments, particularly in latitude. Secondly, when we come down to the order of displacement imposed on the birds, 50–130 miles, the differences to be measured lie within the estimated powers of the organ concerned—the avian eye. Pumphrey (1948) estimates, on the basis of retinal structure, that the limit of resolution would be about 10″ of arc, three times better than the human eye. And the evidence with the latter suggests that the actual maximal acuity is indeed about that forecast on retinal structure. Even with the indirect method of training experiments, Grundlach (1933) found pigeons were resolving down to 23″ of arc at least. We will not be far wrong therefore to accept the structural estimate of a bird's acuity as the threshold. The angular changes it is necessary for the bird to discriminate may be compared with this threshold. Kramer (1953) claims that his birds are orientated within 40 sec. of viewing the sun. This may well be an underestimate, but even so in that short period the sun has moved through an angle 60 times the threshold value. Displacement of 50 miles in latitude results in a change in altitude 260 times the threshold; 50 miles in longitude (at 52° N.) results in a change of azimuth 420 times the threshold. So it is quite possible that the eye could detect the smallest angular changes required of it. We can add that, owing to a lesser contrast between peripheral and foveal vision in birds than in humans, the bird does not have to look directly at an object to follow its movement. Also it suffers no inconvenience from glare. Fifty miles in longitude (at 52° N.) represents a time change of under 5 min., which indicates the limits of

accuracy required in the chronometer. We can say nothing positively about this being a likely limit. The human subject is again likely to have a poorer development of a time sense, yet MacLeod & Roff (1936) found that two human subjects kept in a sound-proof room for 86 and 48 hr. could estimate the passage of time so well that their cumulative errors were 0·8 and 0·9%. For a period of 18 hr., which is about the most that pigeons have been kept shut away from the light in the previous experiments, this would represent an error of 8½ min.!

Though the angular differences and probably the time differences required would seem to lie within the limits of the bird's sensory equipment, an objection is that the bird is not required to compare the position of two observed suns. It must compare the position of the observed sun with that of a 'visualized' sun. This would certainly require a very remarkable spatio-temporal memory, for which we have little definite evidence one way or the other. Certainly pigeons have enduring memories for landscape features, *once* these are learnt (Matthews, 1952*c*; Skinner, 1950). Yet the recognition of one skyline from another depends ultimately on a comparison of all the minute angular differences that distinguish them. So perhaps it is only the unfamiliarity of the conception that makes it difficult to credit the memory and 'visualization' of one striking and familiar feature, the sun arc at home.

The interpretation of differences between observed and remembered sun positions would have to be credited, like the measurement itself, to an automatic innate mechanism. The tests have shown, for example, that a previous experience of longitude displacement is not necessary for accurate navigation from the west. Further (Matthews, 1951*b*), increased experience does not bring much improvement in orientation, but only in the speed and success of actual homing. It has since been shown (Matthews, 1953*a*) that completely inexperienced young pigeons give good homeward orientation from a distant point.



T 20
(cf. T 8 and T 20, Fig. 1)
8/10th cloud
N 17
$\bar{x}$ 70°
σ 48°
P 0·06

Fig. 6. Effect of cloud conditions on initial orientation at right angles to training line.

It is not considered necessary to extend the possibility of sun-navigation by postulating a sensitivity to the polarization pattern of the sky, especially as Montgomery & Heinemann (1952) have been unable to demonstrate such a sensitivity in pigeons by training experiments. With pigeons the only benefit would be in cloudy conditions. Fig. 6 shows that with 8/10th cloud orientation is worse than in sunny conditions, suggesting that the cloud is a considerable hindrance. However, glimpses of the sun would be obtained fairly frequently, and it is possible that the main effect of clouds is an indirect one in these cases—the cloud movement confusing measurement of the sun's movement. Pigeons rarely fly in twilight, none homed after

sundown in the present series, and less than 1% even as late as the hour before. And it is most unlikely that localization of the sun position by this indirect method would be sufficiently accurate for bico-ordinate navigation. At best the bird would get a compass reference for navigation in one co-ordinate (which might be of benefit to night migrants orientating themselves after sundown). This limitation is found in man's own use of the polarization pattern in the Pfund Sky Compass, developed for use in the long polar twilight (Moody, 1950).

We may conclude that although the hypothesis put forward may strain our credulity, it cannot be dismissed out of hand on theoretical grounds. The next step is therefore to examine the accumulated evidence and carry out experiments to see if there is practical support for the proposals.

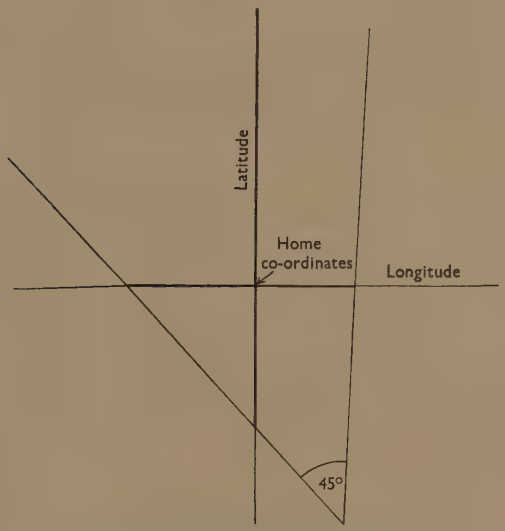


Fig. 7. Diagram illustrating the relative importance of small errors in longitude for a release north or south.

THE ACCURACY OF ORIENTATION VERSUS TIME OF DAY

Consideration of Fig. 5 will show that large errors both in altitude and azimuth would be particularly likely in the early morning (or evening) when the amount of extrapolation is greatest. Conversely, such gross errors are least likely around noon, but here, along the top of the arc, the small errors are more likely to be in azimuth than in altitude. The resultant small errors in longitude are more likely to appear as deviations in the initial orientation when birds are released north or south of the loft. This apparent paradox is resolved by Fig. 7, which shows that in such cases a much smaller margin of error in longitude determination than in latitude determination is permissible if the vanishing point is to fall within the arc

of 45° about the home line. The release point is shown as 20° off true north or south, as this was approximately true of all the cases to be considered. It will also be clear that for releases to the west, small errors in longitude determination will have little effect on the observed orientation.

Combining all north and south releases made in sunny conditions, for the first time at the particular release point and with no attempted interference with sun-navigation, we have 231 observations that may be grouped into two-hourly periods:

	G.M.T. plus/minus 1 hr.			
	06.00	08.00	10.00	12.00
Total	34	75	67	55
Gross errors (>90°) (%)	21	13	12	9
Within homeward arc of 45° (%)	44	40	40	22

Both the predictions made on the basis of the hypothesis are fulfilled, the drop in highly accurate (longitude) orientation around noon is *significant* ($P < 0{\cdot}03$). Fewer western releases are available for the same conditions, totalling seventy-six observations:

	G.M.T. plus/minus 1 hr.			
	06.00–08.00	10.00	12.00	14.00
Total	29	21	26	17
Gross errors (>90°) (%)	21	14	4	12
Within homeward arc of 45° (%)	38	19	23	24

Again there is a fall in gross errors towards noon, but no drop in highly accurate (latitude) orientation occurs there. While it would be interesting to carry out hundreds of further sorties to check that, with sufficient numbers, the gross error trend would be *significant*, limited research facilities are best employed in direct experimental approach.

THE TIME AVAILABLE FOR OBSERVATION

As the reference point must be determined from the sun, the sun must be observed in movement for a short period. The minimum period necessary is unlikely to be demonstrated in the field, as a bird could always take just so much longer in orientation, and small differences in 'orientation time' will be swamped by other irrelevant factors discussed earlier. But it would be reasonable to expect that birds that had a long time to observe the sun would be better orientated than those released immediately on emergence from covering. Kramer (1953) has carried out such an experiment and permitted me a pre-view of his manuscript. Half the birds were kept for 1 and 2 days exposed on the top of a tower, the others within the tower and unable to see sky or sun. On release (in groups), the second lot being allowed half a minute's prior exposure, Kramer claimed that there was no difference in the accuracy of orientation. But the issue was confused by the inclusion of groups of birds that had already flown from that point the previous year,

in sunny conditions on both occasions. We have seen (p. 247) that improvement would be likely to occur in such cases. If we consider only birds released at that point for the first time we can extract the following data for angular deviations from Kramer's diagrams:

	Long exposure	Short exposure
N	5	10
$\bar{x}$	26°	46°
σ	12°	24°

The small number of points available (45 birds were used, but released in groups of 3) makes the application of statistics difficult. The value of 't' between the two groups has a $P = 0{\cdot}1$ only, but the difference is so striking, with deviation and scatter twice as great in the short exposure group, that this cannot be cited as evidence against the hypothesis, and can indeed be accepted as tentative evidence for it.

EXPERIMENTAL USE OF THE SEASONAL CHANGE IN ALTITUDE

Under usual conditions of experiment (p. 252) this will have little effect. But *if* pigeons were unable to make a correction for the daily change at an equinox, and were kept out of sight of the sun for several days, the effect on their orientation would be drastic *if* they were indeed measuring latitude by comparing the sun's altitude with that when last seen at home. It is quite likely that pigeons would not be able to make such a correction for it would have little selection value for them. They do not have migrations and a short breeding season governed by day-length, and hence by sun-height. Under normal racing conditions they are not shut off from the sun for long, and the races involving the longest transport take place near the summar solstice. Nor is there much chance of young birds learning that occlusion of the sun for several days is accompanied by a sharp change in altitude. During the summer months April–September inclusive the percentage of completely sunless days at Cambridge was 4, 10, 11 and 4 in the years 1949–52. Sequences of two sunless days occurred once in 1951, of three once in 1949 and twice in 1950.

A test involving such conditions was carried out in 1951, and as insufficient birds were available for statistical purposes, was repeated in 1952.

Between the third week in July and the second week in September the pigeons were trained in the usual fashion up to 80 miles due west of the loft at Madingley. The experimental release was made on 20 September in both years, at the same point, 78 miles S.S.E. Prior to this the birds had been confined to their usual quarters in the loft, for 6 days in 1951 and for 9 in 1952. Blinds prevented the experimental birds from having any view of the sun or sky during this period, and only indirect light reached them. This was sufficient to make the birds aware of dawn and dusk, and was augmented by artificial light from $2\frac{3}{4}$ hr. after sunrise until $2\frac{3}{4}$ hr. before sunset, so that they could feed and exercise freely. Food was provided regularly, 15 min. after lights on and 15 min. before lights off. Other

pigeons with free access to the sky were kept in the next compartment, separated only by a thick sacking partition, so that their waking/sleeping movements and other activities could be heard by the experimentals. By these methods it was hoped to prevent any desynchronization of such time-keeping mechanisms as the experimental birds might possess. The control birds were in a similar compartment, fed at the same times, but allowed full view of the sun and sky.

The birds were loaded into a screened van after dark on 19 September and taken through the night to the release point, and kept screened until release. By taking the birds south, an increase in the sun's altitude resulted, partially offsetting the seasonal fall during the incarceration:

	1951	1952
Seasonal fall	2° 19′	3° 28′
Positional rise	1° 04′	1° 04′
Net fall	1° 15′	2° 24′

The longer incarceration in 1952, resulting in a more emphatic net fall, was adopted since no extraneous ill-effects had been noted with the shorter period. With no correction for seasonal fall, a false position of home would be obtained by comparing the position in altitude of the sun when last seen with that on the day of release. The false position would have been correct if the sun had 'stood still' and may be termed the 'solstice' home position. In relation to the release point real and false home positions were:

	Latitude (miles)	Longitude (miles)	Bearing
Actual home position	74 N.	21 W.	345°
Solistice home position	86 S.	21 W.	194° in 1951
	166 S.	21 W.	187° in 1952

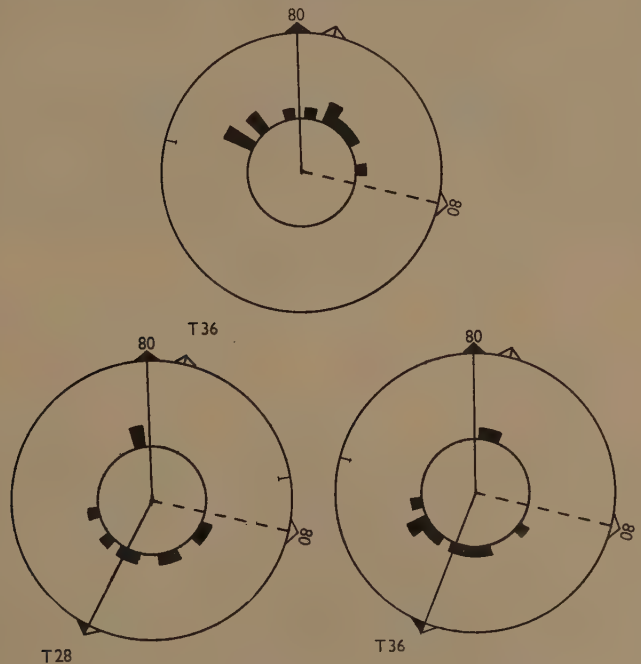
Only experimental birds were used in 1951, in 1952 experimentals alternated with controls throughout the period of releases. The 1951 releases were with 4/10th stratocumulus, the sun in open sky, those in 1952 with a practically cloudless sky ($<1/10$th). The initial orientations are shown in Fig. 8. They may also be compared with earlier releases made at this point with sun, T9, and with overcast, T21, shown in Fig. 1.

The two experimental results are *identical* ($P>0{\cdot}9$) and each *differs* from the controls, with values of $P<0{\cdot}001$. There is thus no doubt that experimental technique has resulted in a radically different orientation. But the values of t_r would not indicate that the scatters are *different* from random. If we seek to confirm this by a χ^2 test, comparing the expected and actual numbers falling into quadrats, we find that $\chi^2=15{\cdot}0$ which has a $P<0{\cdot}002$. So by this test the scatter for the experimentals (the two tests combined since there is no doubt that they are from the same population) is strongly *non-random*. This contradiction is not due to any skewness about the home direction, since t_r about the algebraic mean still gives a *random* value. Therefore the alternative, suggested by inspection, that the scatter

is not about one point but about two, is statistically acceptable. If we therefore exclude the four birds lost in a radically different direction from the others and consider orientation about the solstice home direction, we have:

N	$\bar{x}$	σ	P
18	36°	26°	<0·001

These birds were thus very strongly orientated in the false direction, and this is only explicable on the basis of altitude estimation by comparison, without correction for seasonal change. The four (18%) birds separated from the others appear



	Experimental	Control	Experimental
N	10	13	12
$\bar{x}$	114°	45°	120°
σ	58°	22°	55°
P	0·4	0·03	0·2

Fig. 8. Effect of prior occlusion of sun and sky on initial orientation off training line. N.B. Half-blocked arrow indicates solstice home direction.

to have made just as good a start in the true home direction, though the numbers do not allow statistical justification. It would therefore appear that in this stock of pigeons a few are able to make correction for the seasonal changes in sun altitude,

despite the lack of selective value discussed earlier. It is of interest to note that two of these birds were the outstanding birds of their year, having given fast, consistent returns from all previous releases.

The orientation times of the experimentals were slightly higher than for the controls:

	N	$\bar{x}$	σ
Experimental T28	10	5·4	3·6
Control T36	13	3·8	2·3
Experimental T36	12	4·6	2·5

which would be expected as the former were having their first view of the outside world for some time. They were, however, rather lower than in the case of birds released at this point with overcast (T21, $\bar{x}=7·5$, $\sigma=7·1$), again suggesting that the experimental birds were not dis-orientated, but re-orientated.

It will have been clear from the first section of this paper that the main instrument in the study of pigeon navigation must be the investigation of the initial orientation. So many other factors govern the rapidity and completeness of homing. This is particularly so in the present case, as geographical limitations mean that birds departing in the solstice direction will soon reach the coast and be turned back. There will then be a good chance of such birds finding their way home by prolonged search. Bearing this in mind, we have:

Initial direction	Returned				Lost
	1st day	2nd day	3rd day	Later	
True home ($N=17$)	4	3	2	4	4
Solstice ($N=18$)	0	7	1	6	4

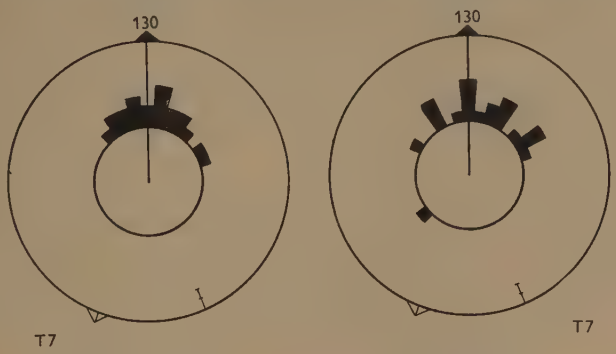
There are thus indications that the re-orientated birds were at a disadvantage over rapid homing, but had not lost the desire to home. The part of the hypothesis dealing with latitude determination can be said to have received very strong support from these experiments. Indeed there seems no other interpretation possible.

EXPERIMENTAL INVESTIGATION OF THE CHRONOMETER FACTOR

There are no convenient seasonal factors in longitude determination from the sun. Thus the sun is always due south at local noon. So any investigation into this part of the hypothesis will have to be aimed at disturbing the working of the inner chronometer, about whose nature we can only guess. Such experiments, at least at first, will have to be of a rather crude nature.

Just as it is probable that over-training in one direction will blunt the ability to navigate in two co-ordinates (Kramer & St Paul, 1951; Matthews, 1951*b*; van Riper & Kalmbach, 1952), it is possible that release at only one time of day might produce an inflexibility in longitude determination. It has been clearly shown (Matthews, 1951*b*, 1952*c*) that there is no question of pigeons using the extremely limited form of orientation given by flying at a fixed angle to the sun, and that

changes in the normal time of release can be taken into account. But the birds subjected to such changes did give rather poorer orientations than did the controls, as will be seen from Fig. 9. The birds in this test had been trained up to 80 miles NNW., each individual being released at the same time of day ($\pm c.$ 12 min.) on the last six occasions. They were then released at 130 miles on the same line, the experimentals 6 hr. earlier or later than normal, the controls at the normal time. The difference is noticeable but only has a significance of $P=0{\cdot}1$. The effect is most marked in the sector close to the home line, in which (see p. 255) small errors in *longitude* determination would have the most effect. Thus the proportions falling within the 45° arc centred on the home line were 62 and 33%, $P=0{\cdot}07$.



Control		Experimental
21	N	21
23°	$\bar{x}$	35°
18°	σ	31°
$<0{\cdot}001$	P	$<0{\cdot}001$

Fig. 9. Effect of radical change in accustomed release time on orientation on training line.

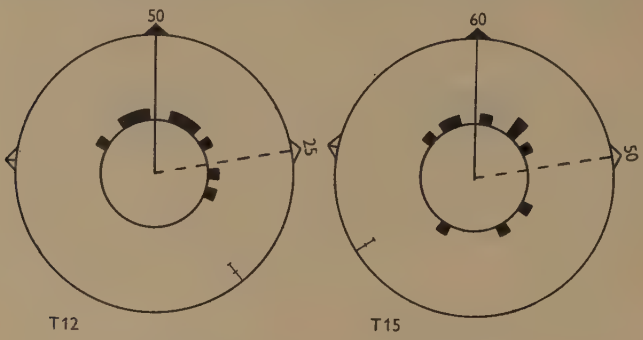
By the same token it would be expected that birds with the most training at a particular time would be the most affected by radical time changes. Fig. 10 illustrates such tests, the birds being released to the west after training to the north at fixed individual times. Those in T 12 had only two such releases, both at 25 miles, those in T 15 had an additional two, at 50 miles. Test releases were 5–7 hr. later than usual at distances of, respectively, 50 and 60 miles. Again the difference is suggestive but not of sufficient significance ($P=0{\cdot}3$), but is most noticeable in the gross errors, which in a western release are more likely to be affected by longitude determination.

These experiments at least suggested that a time mechanism was concerned in longitude determination, so the next step was to attempt to throw such a mechanism out of order. Whatever the ultimate basis of a biological chronometer (? neural) it seemed probable that it would have to be synchronized in some way. Such work as has been done with birds and insects (cited above) concluded that the 'time

sense' had a 24 hr. basis, i.e. animals could be trained to feed once every 24 hr., but not once every 36 hr. Hence likely synchronizing agents are the day/night rhythms of light and darkness and of temperature. Where feeding is very regular this might also be concerned, though this is less 'natural', as foraging in the wild state is spread over many hours.

Birds that had had rather more training to the north than desirable (up to 200 miles) were therefore released to the west after the following treatment:

They were taken from the lofts after dark on 9 September 1952 to the laboratory in a covered van. Half were installed in cages on the roof as controls, being fed regularly at 09.00 and 17.00 G.M.T., having a wide view of the sun and sky to the



Little training		More training
10	N	10
47°	$\bar{x}$	67°
31°	σ	51°
0·05	P	0·4

Fig. 10. Effect of radical change in accustomed release time in orientation off training line.

south and west, and open to temperature changes. The experimental birds were housed in similar cages (allowing *c.* 4½ cu.ft. per bird) in a light-proof room. They were subjected to periods of light and darkness of very unequal length, fed irregularly (total amount of food the same as for controls), and with the temperature fluctuating but little, maxima corresponding to the artificial 'days'. This treatment is shown graphically in Fig. 11, where it is compared with the regularity of the control conditions. The experimental room was not sound-proof, but external sounds were partially muffled and further swamped by the noise of the continuously running electric fan for ventilation. Outside activity was at a minimum during the vacation, and a weekend, with an absence of normal routine, occurred in the middle of the experiment.

The treatment was continued for 6 days, until the night of the 15th, when the birds were loaded on to the screened van after dark and taken to the release point. On arrival the experimental birds were again illuminated, but the controls kept in the dark until after sunrise, when they were also exposed to the internal lighting of

the van. Neither could obtain any view of the outside conditions until the moment of release. The orientations achieved by the two sets of birds, released singly and with controls and experimentals alternating, are shown in Fig. 12.

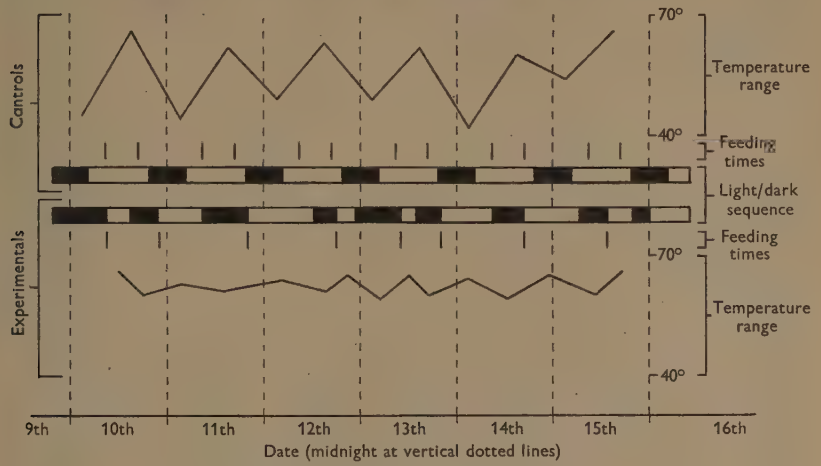
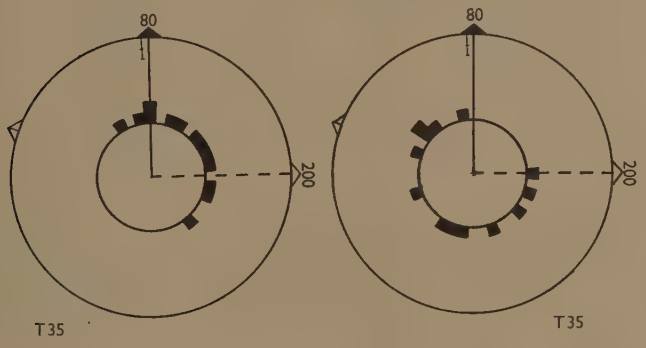


Fig. 11. Diagram of treatment employed in an attempt to desynchronize the chronometers of pigeons.



	Control		Experimental
N	13		13
$\bar{x}$	54°		100°
σ	44°		53°
P	0·09		0·6

Fig. 12. Effect of desynchronizing treatment on initial orientation off training line.

Two *different* scatters resulted ($P=0{\cdot}02$). The t_r test does not differentiate the control scatter from a random one, but a χ^2 test shows it to be strongly *non-random* ($P=0{\cdot}004$). Inspection suggests that this conflict is due to the skewness of the

distribution, and this is confirmed by recalculating t_r about the algebraic mean, when a value corresponding to $P = 0{\cdot}006$ is obtained. The birds were thus showing a conflict between home and training directions, due to the greater amount of training in the latter. The scatter of the experimental birds is *random* both by the t test and by a χ^2 test ($P = 0{\cdot}2$).

The orientation times were:

	N	$\bar{x}$	σ
Controls	13	3·2	1·1
Experimentals	13	4·0	2·2

As in the altitude comparison experiments the experimental figure is slightly higher, and probably for the same reason. Again we have other releases from the same point, in sunny conditions (T 8) and with overcast (T 20)—Fig. 1. The latter also gave a scatter which cannot be differentiated from random, but the orientation time ($\bar{x} = 7{\cdot}8$ $\sigma = 3{\cdot}1$) was *higher* ($P = 0{\cdot}003$) than that for the present experimentals. This suggests that the latter were not completely 'lost', but were in fact re-orientated, the individual directions being different because the irregular desynchronizing treatment had affected the individual chronometers to different degrees. The next step will obviously be to introduce a systematic change in the light/dark rhythm, such as bringing the day forward an hour, artificially. This will be done in the coming season.

As with the altitude experiments, the experimental treatment was apparently inimical to swift returns, the difference being rather more marked:

	Returned				Lost
	1st day	2nd day	3rd day	Later	
Controls	3	5	0	2	3
Experimentals	0	3	2	5	3

This might be due to a more lasting disorganization of the navigation mechanism, also to the fact that birds on a wrong course would have much farther to go from this release point before reaching a sea barrier. The fact that the final returns are the same, again shows that the experimental treatment had not extinguished the desire to home.

It will have been noticed that there is no southwards tendency amongst the experimental birds, as might be expected, due to the sun's seasonal drop, reinforced in this case by previous training. This suggests that latitude determination is intimately bound up with the functioning of the chronometer mechanism, and disruption of the latter will throw out the former as well. In other words, the processes (2) and (3) suggested in the hypothesis proceed as one.

We may conclude that the part of the hypothesis dealing with longitude determination has received strong support from the experimental evidence thus far. But more evidence is necessary before a state of certainty is obtained.

CONCLUSIONS

We have seen that pigeons are capable of bico-ordinate navigation only when the sun is available. With complete cloud cover their orientation breaks down, and this has been demonstrated in a number of different situations. The conclusion that the sun is an essential factor in bico-ordinate navigation is inescapable.

When the simplest hypothesis for sun navigation previously suggested is examined in detail, no over-riding theoretical objections to its validity can be raised. It is therefore acceptable as a working basis for further investigation.

The variation of large and small errors with the time of day lends support to the hypothesis, as does the variation of error with the time available for observation.

The sun-occlusion experiments strongly support the suggestion that latitude determination is based on the observation of differences in sun altitude.

The experiments aimed at upsetting the internal chronometer have produced results supporting the suggestion that longitude determination is based on time differences.

If it is possible in the coming season to obtain a definite re-orientation after treatment aimed at producing systematic error in the internal chronometer, the broad outlines will have been definitely established. It is doubtful if the field technique will be sufficiently sensitive to investigate the details of the hypothesis. So far pigeons have proved unco-operative in a simple apparatus of the type used by Kramer, when they are required to indicate the home direction. It would be nearly impossible to reinforce by training since the bird would have to be taken to a different distant point for each test, otherwise it would simply tend to learn a compass direction. One possible, though expensive, solution might be the use of a planetarium in which the complete movement of the sun could be reproduced. The pigeons could then be trained to proceed in different directions to food, corresponding to the changes produced in the 'sun's' co-ordinates. If the apparatus were large enough to allow the birds to fly, the results might be more reliable, for it is always possible that a complicated behaviour pattern, such as sun-navigation, will only be triggered off in the appropriate situation, in the air.

It will be more than doubly conclusive if the more detailed analysis of sun-navigation can be repeated in another species. The excellent Manx shearwater holds out the most possibilities in this direction, though many features of its life may make it more difficult to confuse this bird by experimental treatment.

Lastly, the whole fascinating problem of the accuracy and physical basis of the 'time sense' has barely been touched, and calls for much more detailed research in its own right, as well as in connexion with this particular problem.

SUMMARY

1. The accumulated data of a long series of homing experiments carried out with young pigeons is examined in statistical detail.

2. Bico-ordinate navigation can only be demonstrated in sunny conditions, and with overcast skies the birds scatter at random. This holds for a number of different experimental situations.

3. The hypothesis that pigeons use a form of sun-navigation, deriving latitude and longitude from the sun position alone is re-examined in detail. None of the requirements are outside the theoretical range of the organ concerned, or beyond the bounds of possibility.

4. Examination of the errors made under sunny conditions shows that their nature and variation are as expected on the basis of the hypothesis. So also is the variation in error with the length of time of observation.

5. By excluding sun and sky for a number of days before release a re-orientation away from home was obtained. This could only be explained if the birds were failing to correct for the seasonal variation in the sun's altitude, *and* deriving their latitude from altitude measurements.

6. Radical changes in accustomed release times produced some increase in longitude errors.

7. After treatment aimed at desynchronizing any internal chronometer a disorientation was obtained in the field. It is therefore very probable that longitude determination is on a time basis, as proposed in the hypothesis.

8. Future lines of research in this problem are indicated.

I am much indebted to Prof. James Gray for his interest in and facilitation of this research, and to Dr W. H. Thorpe for his continued encouragement. Dr R. G. Newton was an invaluable guide in matters statistical. Messrs P. Cope, L. Duke, A. Leader and H. Wilson played an essential part in breeding and training birds for some of the tests. The maintenance of the loft at the Ornithological Field Station was possible with the unsparing co-operation of Dr R. A. Hinde, and the routine work was ably carried out by Mr G. G. Dunnett. Dr R. J. H. Brown permitted the examination of his yet unpublished material, and I should particularly like to thank Dr G. Kramer of Wilhelmshaven for much valuable discussion in person and by letter, and for allowing me a pre-view of his latest paper in typescript. The Botanic Gardens, Cambridge, provided local meteorological data.

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THE ORIENTATION OF UNTRAINED PIGEONS: A DICHOTOMY IN THE HOMING PROCESS

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INTRODUCTION

It had been shown (Matthews, 1951*b*) that reasonably accurate orientation and homing could be obtained with young pigeons after a minimal training up to 25 miles. Recently Kramer & St Paul (1952) and Kramer (1953) have obtained similar results with older pigeons trained up to 10 miles only. Training thus appeared to be rather less essential than had been supposed, and to investigate this further, experiments with completely untrained young pigeons were required.

Table 1. *Results of previous experiments with untrained pigeons*

(Data from Rivière, 1929 (R); Gibault, 1930 (G); Heinroth, 1941 (H); Lockley, 1942 (L); Platt & Dare, 1945 (P)).

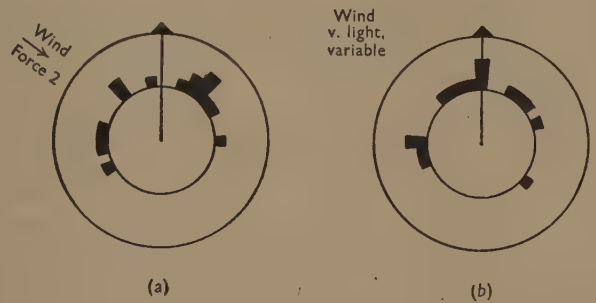
Distance (miles)	Released	Returned	Source
34	14	1	H
58	7	7	R
62–93	4	—	H
80	14	—	P
95–102	7	2	R
118	10	5	G
130	4	1	L
136	16	5	G
155	59	1	H
186	10	3	G
215	5	—	L
250–597	7	—	H

Anecdotes of untrained pigeons homing over considerable distances are plentiful, but it is almost impossible to obtain precise details. The very fact that such occurrences are particularly remembered is evidence of their rarity. A number of authors have reported on deliberate releases of untrained pigeons at distances over 25 miles from home, giving the results shown in Table 1. Few birds returned, and those slowly with only four returns on the day of release. Most reports of lost birds came from near the release point (31), but five had gone from 50 to 120 miles, all but one deviating less than 45° from the home line. There is thus a suggestion of navigational ability in these untrained pigeons, but as they stand the results can easily be fitted into a theory of random search for known landmarks such as that discussed by Wilkinson (1952). A considerable variation in the results obtained by different

workers is noticeable. Unfortunately, no details were given of the initial orientation of the birds after release, now recognized to be the keystone of any investigation of their navigational ability, and this could only be obtained by further tests.

MATERIAL AND METHODS

Young pigeons were reared at the Ornithological Field Station, Madingley, Cambridge, from the same breeding stock as those used in the main field experiments (Matthews, 1951*b*, 1953), being the third brood raised in the season. They were kept under normal conditions, and permitted the usual amount of almost daily exercise flights from the loft. The first experimental release (UT 1) showed the birds unwilling to fly far when thrown up for the first time, and so for subsequent tests the birds were previously given several experiences of spending nights in the basket, and of being released singly, all within the home compound, less than 150 yards from the loft.



Aged 3/4 months (UT 4)		Aged 8/12 months (UT 2/3)
16	No. of birds	16
55°	Mean angular deviation	51°
32°	Standard deviation	42°
0·03	Probability	0·03

Fig. 1. The initial orientation of untrained pigeons. Explanation: the vanishing points are grouped into 10° sectors; solid arrow, home direction.

Release points were in open country with good all-round views. The birds were taken there in covered baskets and kept covered until release. In all cases this was in bright sunshine, with cloud 4/10th or less, the birds thrown up singly and followed with 16 × 40 binoculars until out of sight. The bearing of the vanishing point (Initial Orientation) and the time between release and vanishing (Orientation Time) were noted. The scatter diagrams (Fig. 1) obtained by plotting the vanishing points relative to the home direction were tested for statistical significance by comparing the mean angular deviation (regardless of sign) with that of a random distribution by means of a *t* test, as described in Matthews (1953).

RESULTS

Five pigeons, aged 13/14 weeks, were released singly at a point 78 miles distant, home bearing 345°, on 20 September 1951. This site had been used in a number of previous experiments, and scatter diagrams for these—T 9, 21, 28, 36—are available for comparison in Matthews (1953). In this first release (UT 1) no evidence on orientation was obtained. Four birds came down in distant trees. Another release was made at this point on 20 September 1952 (UT 4), using sixteen pigeons aged 13/16 weeks, which had had the basket training described earlier. These flew well and produced the initial scatter diagram shown in Fig. 1(*a*). This shows a definite homeward orientation, the *t* test giving $P = 0{\cdot}03$.

Not one of the twenty-one birds from these two releases returned home. The only one reported elsewhere had flown to a point 67 miles away only 22° off the home line, its error in latitude being only 7 miles. Too much emphasis cannot be laid on this, as the bird was not secured until 6 months after the release, although there is some evidence that it might have been present for a long time. Allowed to breed at Madingley it was released without further training on 16 September 1952, 65 miles WSW. Nothing further has been heard of it, and one cannot assume that it was a particularly gifted bird.

As it was possible that the distance was too great for the young, untrained birds, other tests were made with older birds, also untrained, at a shorter distance. Six cocks aged 31/33 weeks, which had been prevented from mating, were released at 50 miles, home bearing 022°, on 13 April 1952 (UT 2). An excellent orientation resulted, but again there were no returns. Birds with an even greater incentive to get home were therefore used in the next test (UT 3), eight cocks and four hens which had reared two broods of young and were starting a third, aged 39/52 weeks. To avoid any topographical bias, they were released at a similar distance to the NNW. instead of to the SSW., distance 53 miles, home bearing 158°. Two were 'treed' but the others gave an orientation similar to that in UT 2, and can be considered together with that in Fig. 1(*b*). The homeward trend is again statistically significant, $P = 0{\cdot}03$. Indeed the two distributions (*a*) and (*b*) are so similar that the chances of their being drawn from the same population are $P = 0{\cdot}8$, i.e. they can be considered together. The chances of such an overall orientation being produced by non-orientated birds are correspondingly lower, $P < 0{\cdot}001$. We can therefore have no hesitation in asserting that untrained pigeons show a homeward orientation when released in unknown country.

Even with the strong motivation presumed in UT 3 only one of the twelve birds returned home, being back early next morning. This cock was subsequently trained in the normal way and proved to be above the average in performance.

The results obtained with these untrained birds can be compared with those of birds which had had various amounts of training, as in Table 2. Scatter diagrams for the trained birds are figured in Matthews (1953). Training was by increasing steps in one direction to the distance shown, the birds then being released roughly at right angles to this training line. Those in T 9 were released in the opposite direction,

but at right angles to the previous critical test, T 8. Kramer's (1953) training was in all directions, so that the area flown over by his birds was 20 miles wide. His birds were released in small groups (all the others being singles) and include only those released after a short exposure to the sky, i.e. in comparable circumstances to the other tests. He gives no details of orientation times, and the proportion homing cannot be taken into account since it would be misleading. The releases of the untrained birds in his case were closely interspersed with those of birds that had already flown from the same point, and there is a strong possibility of the untrained birds joining with these potential guides out of sight of the release point.

Table 2. *Performances of untrained and trained young pigeons*

Test	Distance (miles)	Age (months)	Training (miles)	No.	Orientation (°)		Orientation time		Returns (%)
					Mean	S.D.	Mean	S.D.	
UT 1/4	78	3/4	Nil	21	55	32	5·6	2·4	Nil
UT 2/3	53	8/12	Nil	18	51	42	5·7	2·6	6
Kramer	200	13/19	10	10	46	24	N/A	N/A	N/A
T 12	49	4/5	25	10	47	31	5·8	4·2	70
T 15	62	4/5	50	10	67	51	4·1	2·5	80
T 36 C	78	4/6	80	13	45	22	3·8	2·3	76
T 8	79	5/6	130	47	45	30	3·3	1·1	87
T 9	69	5/6	130+T 8	33	45	33	4·4	2·3	94
T 35 C	80	6	200	13	54	44	3·2	1·1	77

From Table 2 we can conclude that:

(1) The accuracy of orientation shows no significant improvement with training, the difference between the worst untrained and the best trained having $P=0{\cdot}3$. This is not to be confused with the undoubted improvement that does occur *on* the training line due to a blunting of true navigational ability by constant release at one time and in one direction. Overtraining in this way may lower the accuracy of orientation off the training line as, respectively, in T 15 and T 35, as discussed in Matthews (1953).

(2) The orientation time becomes reduced with training, the fall being sharpest after the 25-mile stage, and the extreme values differing significantly.

(3) The proportion of returns mounts very rapidly with a small amount of training. Thereafter there may be some further improvement, but this is not consistent.

These conclusions suggest that there is an essential dichotomy in the homing process, namely between the factors governing initial orientation, which are completely innate, and those governing the actual return, which are largely dependent on individual experience. Orientation time falls in the latter group, and it is probable that in the good conditions of these tests it bears little relation to the orientation process, although when the latter is disturbed (Matthews, 1953) there is a corresponding rise in the orientation time. A less definite term, such as 'Time in sight' would probably express this measure of performance better.

The nature and basis of the process producing initial orientation has been examined in another paper (Matthews, 1953), in which the conclusion was reached that a form of sun navigation, as proposed earlier (Matthews, 1951*a*, *b*), is probably concerned. This would be a highly complicated process which certainly could not be learnt by an individual, and so is in accord with the demonstrated innate nature of the orientation process. The young birds of course had no opportunity to 'practice' such a mechanism in their restricted exercise flights from the loft. The slight improvement in orientation with experience is probably due to a weeding out of imperfectly equipped birds, and to less loitering near the release point which means less chance of a bird being lost on evolutions not connected with orientation.

The factors which are concerned with the actual successful return, and which are developed by individual experience can probably be placed in three groups:

(*a*) *Psychological.* Training gives a bird the experience of flying considerable distances back to the loft, implanting the information that flying long enough in the direction indicated by the orientation mechanism will produce the desired result. The 'confidence' thus imparted also increases the willingness to start out alone (witness the decrease in 'orientation' time) and makes the bird less likely to be diverted from its course by joining strange flocks of pigeons. The awareness that food will be waiting at the end of the journey will also prevent foraging and possibly fatal delay.

(*b*) *Topographical.* Young untrained pigeons do not go far from the loft on exercise flights; repeated observation makes it doubtful if they explore more than a mile radius. Certainly it is possible to lose them at short distances on the initial training steps—$\frac{1}{2}$, 1, 2, 4, 6 miles—eighteen young birds disappeared from these releases in 1951 and 1952, 13% of the total. With such a small 'target' the chances of even a well-orientated and 'confident' bird reaching home from 50 or 80 miles would be understandably slight. Training that increases the 'target' width to 20–30 miles brings it within the limits of accuracy that could be postulated for sun-navigation.

(*c*) *Physical.* The development of strong muscles and stamina would be hastened by training, but this is less likely to be critical. The untrained birds at 12 months were certainly stronger than the trained youngsters a third the age.

It was demonstrated earlier (Matthews, 1951*b*) that there were wide individual variations in homing ability. This, of course, only confirmed the opinions of practical fanciers who treat their champion racers as individuals—making allowance for one always flying itself to exhaustion, another always taking its time, for one homing faster to eggs, another to young, and so on. Indeed the multiplicity of their schemes for success reflects the highly complicated nature of the problem. Statistical confirmation of this point, and of the contrast with the relatively unitary nature of the orientation process, can be obtained from a consideration of the individual case histories of the birds used in previous tests. Details of initial orientation, time in sight and homing success are recorded for each sortie flown. The individual performance over the season can be reduced to numerical indices

by awarding arbitrary points according to the method described by Matthews (1952*b*). The justification for the values allotted and other features of the scheme is given therein; here the basic details are set out baldly in Table 3. Limiting consideration to those birds which made at least two individual sorties from 50 miles or more in sunny conditions, but otherwise taking all available data, we have indices for 104 birds, covering 482 sorties.

Table 3. *Arbitrary system of points awarded for performances*

Initial orientation

Deviation from home ...	0–30°	–45°	–60°	–90°
On training line	20	15	5	2
Off training line	40	30	10	4

Returns

Back on ...	1st day, speed above average	1st day, speed below average	2nd day	3rd day	Later
On training line					
50 miles	20	10	5	2	1
80 miles	40	20	8	3	2
Off training line					
50 miles	40	20	15	6	3
80 miles	80	60	10	10	5

Notes. (1) repeat performances rated at half the values shown; (2) allowance for tests missed by bird's own shortcomings made by adding percentage of possible points to total gained; (3) resultant totals scaled down so that best performance in each section rates 100.

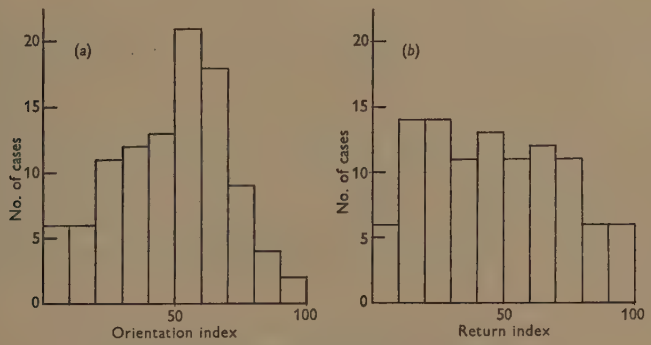


Fig. 2. Frequency distribution of indices of orientation and return.

The frequency histograms for the resultant indices of orientation and return are shown in Fig. 2(*a*), (*b*).They have mean values close to the medianal value, 47 in each case, indicating an adequate sample. But the distributions are very dissimilar ($P=0{\cdot}01$). That of the orientation index approaches the form of a normal curve which would result if we were dealing with a single quality (or group of interdependent qualities) varying about its mean. On the other hand, that of the return

index departs strongly from normal and is of the form that would be expected if we were measuring the effects of a large number of independent factors.

The relation between the two indices is shown by the contingency table (Table 4). There is a general overall association between them ($P = 0{\cdot}01$) as would be expected with qualities which both contribute to homing success and thus likely to be selected out together to some extent. At the same time, since such a large number of factors is concerned, the chances of their being equally developed in one bird are small, and the correlation is by no means strict. It is strongest in the case of birds showing poor orientation ability, such birds generally producing poor returns. With good or moderate orientation ability the factors governing the return assume the greater importance. We have a further indication here that the orientation mechanism need not be of phenomenal accuracy, its main function being to impart a homeward trend to the flight from unknown country, home being pin-pointed by pilotage rather than navigation.

Table 4. *Relation of indices of orientation and return*

Return \ Orientation	Index	0–33	–67	–100
	0–33	18	17	5
	–67	5	23	11
	–100	5	14	6

It would clearly be desirable to follow up these individual variations in ability on a genetical basis, but this would be extremely difficult, if not impossible, with such a relatively slow breeding animal, quite apart from difficulties of measuring the factors under consideration. There is no doubt that the selection imposed by racing the birds, and by 'intuitive' cross-breeding, can improve their homing qualities. The differences between performances of birds given the same training and tests, but coming from separate stocks was demonstrated earlier (Matthews, 1951*b*), and that conclusion is confirmed by the more refined method of assessment used in the present paper. Thus the birds used in 1949 from Duxford (27) and from Sawston (25) which had sufficiently complete case-histories gave distributions of indices as follows:

	Orientation		Returns	
	Duxford	Sawston	Duxford	Sawston
Mean	49	67	52	67
S.D.	25	21	28	20

The Sawston stock gave a higher mean value and less variation in both cases, particularly in the (innate) orientation process, the differences being significant with $P = 0{\cdot}009$ and $0{\cdot}04$ respectively.

DISCUSSION

Now that the very different natures of the orientation and homing processes, and their hereditary basis, have been demonstrated, the reasons for conflicting results obtained with pigeons by various authors will be clear. Many doubtless worked with birds of inferior stock, often just to test some private theory, and without taking the necessary step of getting to know one's experimental animal. Also attention was concentrated on the returns rather than on the initial orientation. The basis of the navigational process is the problem of the greatest theoretical interest (although it may yet have practical implications), and it is now clear that it will be solved by investigation of the orientation behaviour. It is even doubtful if observation of the homeward track by aircraft or more subtle devices will be as useful. The amount of training given to pigeons before an experimental release can be much reduced, *provided* the birds are derived from a stock with homing qualities highly developed. This will reduce the amount of work for a given result, and also reduce the number of birds lost on routine training, and thus 'wasted'.

It is at least probable that a similar dichotomy in the homing process will be present in wild birds, the 'training' being provided by migration experience. The only comparable experiment to those described for pigeons was that by Ruppell & Schein (1941) with young starlings. These were prevented from migrating and kept over winter in an aviary until they began breeding. Taken to a point 71 miles away they all failed to home, although starlings similarly treated after several return migrations homed well. The vital orientation data are lacking however, as the birds simply flew into trees on release. It must be emphasized that for parallel studies on navigation in wild birds, a species that has suitable release behaviour must be used. Ideally this is a bird that is unwilling to settle (i.e. released over unfavourable terrain) and which depends on flapping flight in these circumstances (i.e. not soaring birds).

Such studies as have been made with non-migratory birds (e.g. Creutz, 1949) have indicated a very restricted homing ability, but again adequate orientation data are lacking. Matthews (1952*a*) showed that the Herring Gull, a restricted nomad, was a much poorer homer that the migratory Lesser Black Back Gull, but that (statistically insufficient) traces of homeward orientation could be found in the former.This raises the speculative point of whether an orientation mechanism could remain in a population not making active use of it. It must be remembered that migration is a somewhat flexible habit. Lack (1943) showed that birds of a single species in one area varied in their migratory habits, and differences in this respect between local groups of a species are well known, e.g. Herring Gulls in Britain and on the Continent. Moreover, the Lesser Black Back Gull in Britain has in the last 100 years (say in some twenty generations) changed from being mainly resident throughout the year to a total migrant, and is now showing signs of reverting to its former status. Even with such flexibility a navigation mechanism that had no other function might well be eliminated in a sedentary population. But if the mechanism was based on an organ, such as the eye, and on physical data, such

as the sun's co-ordinates, which both play other vital roles in the animal's life, then it might well continue, dormant, ready to become effective if circumstances suddenly demanded it.

SUMMARY

1. It is demonstrated that young pigeons without any previous training show a definite homeward orientation at a distance, yet home very badly.

2. The results are compared with those of birds with varying amounts of training. An essential dichotomy in the homing process is revealed, between the orientation process which is innate and the process of return which is governed by factors developed by individual experience.

3. The further contrast between the unitary nature of the orientation process and the multiplicity of factors concerned in the return is supported by statistical consideration of the individual variation in ability.

4. The practical and theoretical implications of these conclusions are considered.

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FURTHER STUDIES ON IONIC REGULATION IN MARINE INVERTEBRATES

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I. INTRODUCTION

Marine invertebrates, although permeable in some degree to the ions of sea water, show a variable amount of ionic regulation in their body fluids while maintaining the same total concentration of ions in these fluids as the external medium. In a previous study (Robertson, 1949), based on the examination of twenty species belonging to five phyla, it was shown that characteristic patterns of ionic regulation existed in different groups, varying from almost complete ionic equilibrium between internal and external media in echinoderms to regulation affecting usually every ion in the decapod crustaceans. Data on other sixteen invertebrates are discussed here, and certain conclusions are reached based on both studies.

Procedure and chemical methods were the same as in the previous paper (Robertson, 1949). Ionic regulation was there defined as 'the maintenance in a body fluid of concentrations of ions differing from those of a passive equilibrium with the external medium'. Accordingly, an analysis of a body fluid as first obtained from an animal was compared with an analysis of the same body fluid after it had been dialysed in a collodion sac against the original sea water, the latter process giving figures for physico-chemical equilibrium, in which there is formation of calcium-protein complexes and a Donnan effect with protein-rich bloods, such as those of decapod Crustacea and cephalopod Mollusca. The echinoderm, sipunculid, lamellibranchs and nudibranch gastropod all had very low protein contents, below 1 g./l., and in these cases dialysis was unnecessary. Differences from ionic equilibrium are probable only if the concentrations of ions in the original body fluids differ from those of sea water or dialysed plasma by the following percentages: Na 0·6, K 2·6, Ca 1·5, Mg 1·8, Cl 1·1, SO_4 1·2. In the determination of the Donnan ratios, and in some of the potassium and magnesium analyses where the concentrations in the body fluid and in sea water were very similar (e.g. in *Holothuria*), duplicate or triplicate estimations were made to increase the accuracy.

The chlorinity of the sea water in which the animals were kept was 19·5‰ at Plymouth (*Maia*, *Sepia*: sea water from beyond breakwater), 22·5‰ at Naples (*Holothuria*, *Ostrea*, *Mytilus galloprovincialis*, *Dromia*, *Pachygrapsus*, *Squilla*: aquarium sea water), and 17·6–18·3‰ at Millport (the remaining animals).

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II. ECHINODERMATA AND SIPUNCULOIDEA

Ionic exchange in holothurians may be considered to take place between the perivisceral fluid and sea water across the integument and across the epithelium of the respiratory trees. Koizumi's (1932) analysis of *Caudina chilensis* indicated that in this holothurian the perivisceral fluid and surrounding sea water were practically in complete equilibrium, although potassium was always slightly higher (+7%). He found also that the animal responded to changes in the values of the external ions by corresponding alterations in the internal ions, and later (Koizumi, 1935*a*, *b*) that the isolated body wall showed complete permeability. Bialaszewicz (1933) found at Naples that *Holothuria tubulosa* had higher values of chloride (103·5%), potassium (110%) and calcium (109%) than sea water (he did not estimate sodium), but his chloride figure suggests that the animals were imperfectly equilibrated with the sea water in the aquarium, and his methods of analysis for potassium and calcium were not very accurate (see Robertson & Webb, 1939).

Table 1. *Echinodermata and Sipunculoidea*

Coelomic fluid	Concentrations as percentage of sea water values						mg./ml.	
	Na	K	Ca	Mg	Cl	SO_4	Protein	H_2O
Holothuria	100·8	103·0	101·8	103·8	100·5	100·3	0·7	982
Phascolosoma	103·8	109·7	104·3	68·6	98·5	91·3	0·9	983

The mean of two closely agreeing analyses of *H. tubulosa* are shown in Table 1, and it is evident that almost complete equilibrium obtains between the perivisceral fluid and sea water. A third specimen, apparently as healthy as the others, was found on dissection to be without a gut, thrown off no doubt on capture 4 days previously, as may happen in these animals. The analysis of its perivisceral fluid showed no regulation of magnesium, but was otherwise identical with the other analyses, thus perhaps suggesting that the gut itself was playing no major part in the slight regulation of potassium which presumably is effected by the epithelium of the integument.

An analysis of the body fluid of an unnamed species of the sipunculid *Phascolosoma* was made by Steinbach (1940) who was concerned chiefly with the electrolytes in the muscle. Expressed as a percentage of the values in sea water his figures for sodium, potassium, calcium and chloride come out as 86, 422, 117 and 84% respectively. It seems quite clear from the evidence given below that the animals must have been in equilibrium with sea water of a salinity about 15% lower than that given by Steinbach. The figure for potassium is open to grave doubt, as so far no marine invertebrate has shown regulation of potassium beyond 220% (Robertson, 1949; Prosser, 1950), and that exceptional value was in an active animal (*Loligo*) with well-developed excretory organs concerned in ionic regulation.

The analysis of three specimens of *P. vulgare* in Table 1 shows regulation of all ions, but the potassium concentration in the body fluid is less than 110% of that in

sea water. Magnesium is markedly decreased, and the slight rise in sodium seems to have been necessary to maintain osmotic equilibrium. Total ionic concentration comes to 1·055 g. ions/kg. water, compared to 1·065 for the sea water. To the former must be added 3 or 4 mg. ions for bicarbonate which was not determined, and this will bring the respective concentrations within 1% of each other. Osmotic equilibrium within 1% was also found in Bottazzi's (1908) freezing-point determinations on the allied *Sipunculus nudus*. An incomplete analysis of *Sipunculus* by Bethe & Berger (1931) showed a reduction in magnesium to about 70% of the sea-water value. In contrast to the polychaetes *Aphrodite* and *Arenicola*, which show increases in potassium and sometimes decreases of sulphate with no regulation of the other ions (Robertson, 1949), the sipunculid *Phascolosoma* shows marked diminution in magnesium with a slight increase in sodium.

III. CRUSTACEA

The decapod crustaceans are a group showing pronounced ionic regulation, the mechanism of which consists in selective excretion of ions from the blood by means of the antennary glands and a controlled uptake of ions through the gills. Owing to a high protein content, a passive equilibrium between the plasma and sea water across the gills (if it existed) would result in higher concentrations of cations and lower concentrations of anions in the plasma, in addition to a much higher calcium content because of the formation of a calcium-protein complex. Ionic regulation over and above such a possible passive equilibrium is shown by the data in Table 2.

Table 2. *Decapod and stomatopod Crustacea*

	Concentrations in plasma as percentage of concentration in dialysed plasma						mg./ml.	
	Na	K	Ca	Mg	Cl	SO_4	Protein	H_2O
STOMATOPODA								
Squilla mantis	111	129	108	32	101	82	65	916
DECAPODA								
Paguridea								
Eupagurus bernhardus	105	130	137	49	96	135	69	926
E. prideauxi	102	156	126	76	98	129	80	916
Dromiacea								
Dromia vulgaris	97	120	84	99	103	53	37	947
Oxyrhyncha								
Hyas araneus	102	132	106	93	102	104	29	952
Maia squinado	100	125	122	81	102	66	53	931
Brachyrhyncha								
Portunus depurator	105	134	111	47	97	78	37	951
P. puber	110	147	120	41	101	83	54	936
Pachygrapsus marmoratus	94	95	92	24	87	46	35	951

Pachygrapsus marmoratus is peculiar in having all its ions below equilibrium values, and for its low magnesium and sulphate concentrations. The pooled plasma of twelve male specimens had a total ionic concentration of 1·163 g. ions/kg. water compared with 1·350 for sea water (1·353 including bicarbonate), equal to about 86% of sea water, a finding which may be compared with the freezing-point determinations of Schwabe (1933) $\Delta = 2{\cdot}11$ and 2·33° C. respectively, the blood

being equivalent in concentration to approximately 91% sea water. Hypotonicity of other members of the grapsoid group when living in sea water is well established (*P. crassipes*—Baumberger & Olmsted, 1928; Jones, 1941; *Leptograpsus* and *Sesarma*—Edmonds, 1935; *Eriocheir*—Conklin & Krogh, 1938).

Portunus depurator and *P. puber* belong, like *Pachygrapsus* and *Carcinus*, to the tribe Brachyrhyncha. Analysis of the plasma from five male specimens in each case revealed a regulation similar to that of *Carcinus maenas* (Webb, 1940), although the concentrations of magnesium and sulphate are somewhat higher. Margaria (1931) compared the vapour pressure of the blood of these two species with sea water, finding both fluids isosmotic within 2%. This has been confirmed from the present chemical data. The total concentration of the plasma of *Portunus depurator* was 1·070 g. ions compared with a sea-water figure of 1·091, but an additional 5 or 6 mg. ions must be added to the first figure for bicarbonate. *P. puber* plasma was slightly hyperosmotic, 1·105 g. ions compared to 1·082 g. ions.

Dromia vulgaris is peculiar among decapods in showing a low sulphate concentration (53%) coupled with a high magnesium (99%) and reduced calcium (84%). At first sight a sodium concentration of only 97% of the value after dialysis is incompatible with osmotic equilibrium. However, the total ionic concentration in the plasma is 1·336 g. ions/kg. water, compared with a figure of 1·354 for the surrounding sea water, and this slightly lower sodium concentration can be anticipated from the reduced sulphate. In a mixture of electrolytes corresponding to sea water, a reduction of the sulphate concentration to a half and replacement by chloride will lower the total concentration of ions by 0·9%, increase the chloride to 103% and decrease the sodium to 97%, while maintaining complete equilibrium, all these changes arising from the poor osmotic properties of sodium sulphate (Robertson, 1949). The figures for *Dromia* refer to the mean of eleven crabs, six males and five females. Little difference was found between the sexes, except in potassium. The separate figures are (males first) Na 97, 96; K 113, 126; Ca 82, 86; Mg 98, 100; Cl 104, 103; SO_4 54, 52.

The ionic regulation shown by the two spider crabs *Hyas araneus* and *Maia squinado* is not dissimilar, although sulphate is markedly reduced in *Maia*. They maintain relatively high magnesium concentrations compared with the species of Brachyrhyncha. *Hyas* plasma, which came from six male specimens, appears to be almost 2% above equilibrium, 1·102 g. ions/kg. water compared with 1·082 for the sea water in which it was living.

The hermit crabs *Eupagurus bernhardus* (six males) and *E. prideauxi* (twelve females) are unique among decapods in accumulating considerable quantities of sulphate, and correlated with this have lower chloride concentrations to maintain the balance of anions. Perhaps part of the inorganic sulphate is of endogenous origin, but this point has not been investigated. Both species had high protein contents, the highest so far found in intermoult decapods. Osmotic equilibrium within 1% is indicated by the g. ions/kg. water, 1·094 in the plasma of both species compared with sea waters of 1·104 and 1·095 in the cases of *E. bernhardus* and *E. prideauxi* respectively.

Analysis of the pooled plasma from four female specimens of the stomatopod *Squilla mantis* showed regulation of the same type as is usually found in decapods, namely, accumulation of cations except magnesium which is markedly reduced, and some diminution of sulphate. Compared with a concentration of 1·359 in the sea water of Naples aquarium the plasma had 1·380 g. ions/kg. water.

In Table 3 are set out comparative analyses of the plasma and the secretion of the antennary glands in a large male *Maia*. Although the secretion is isosmotic with the plasma, selective excretion by the glands of every ion except potassium is shown by the data. While the higher levels of magnesium and sulphate in the secretion are correlated with plasma values much below those of equilibrium, and a lower level of chloride with a value slightly above equilibrium, there is no such correlation in the case of calcium. The calcium excretion is tending to reduce the blood calcium, since at 99% of the plasma level it is 9% above the ultrafilterable calcium. However, the plasma calcium of this specimen is about 16% higher than the dialysis value.

Table 3. *Antennary gland secretion of* Maia squinado

	mg./g. water						mg. ions/ kg. water
	Na	K	Ca	Mg	Cl	SO_4	
Plasma	11·49	0·498	0·558	1·098	20·19	1·428	1157
Secretion	11·45	0·486	0·550	1·178	19·79	3·064	1163
Sea water	11·27	0·408	0·431	1·359	20·31	2·834	1172*
Secretion as % plasma	100	98	99	109	101	214	100
Plasma ultrafiltrate† as % plasma	98	98	90	96	102	104	100

* Includes 2 mg. ions HCO_3.
† Calculated from dialysis experiments, using the mean Donnan ratio except in the case of calcium.

Potassium of the blood is above that of sea water or of dialysed blood, and the antennary gland secretion is tending to maintain the high value since its potassium is at the level of an ultrafiltrate of the plasma. A level of sodium ions in the secretion higher than in an ultrafiltrate of the plasma would tend to lower the sodium of the plasma, which, however, is in approximate dialysis equilibrium with the sodium of the sea water. This relatively high sodium of the secretion would seem to be necessary to balance the high level of divalent sulphate ions. It can be calculated that if sulphate had not been selectively excreted, that is, had been 104% instead of 214% of the plasma value, the sodium figure would have been about 2·8% below its value in Table 3 to maintain osmotic equilibrium with the plasma.

Selective excretion is only one factor in ionic regulation. Equally essential is the controlled uptake of water and ions which occurs simultaneously with the formation of antennary gland secretion. In the *Maia* specimens of Tables 2 and 3 absorption of potassium, calcium and chloride must take place against concentration gradients, although magnesium and sulphate may be able to enter the blood in accordance with the diffusion gradient. Sodium ions and water must also be absorbed, probably actively, completing the final constituents of a fluid, isosmotic with the plasma, which replaces the isosmotic secretion of the antennary glands. The data in

Table 3 amplify those of Bialaszewicz (1932) for the same species, although there are a few discrepancies between the two sets of results. On a volume basis he found in two cases potassium in the secretion to be 17·9 and 17·0% below that of an ultrafiltrate of the blood, a more usual finding than the present non-selective excretion (cf. Robertson, 1949). Calcium was 22 and 44%, and magnesium 39 and 51% above the values in ultrafiltrates, while one value for sulphate was 45% higher. Bialaszewicz claims, however, that a proportion of certain of the elements is bound to colloids, 3·8% of the potassium, 16·4% of the calcium and 12·8% of the magnesium, only chloride and sulphate being completely diffusible across a collodion membrane. He is probably in error here with respect to potassium and magnesium, since no binding of these ions has been found by Webb (1940) or Robertson (1939, 1949) in crustacean blood, although a Donnan ratio of 1·01–1·03 depending on the protein content has been found for all the cations except calcium. In a *Maia* specimen with 41 g. protein/l. I have found the ratio magnesium plasma/magnesium ultrafiltrate to be 1·065, giving a Donnan ratio of 1·03, and the ratio for plasma dialysed against sea water to be 1·051, the agreement between ultrafiltration and dialysis being fairly good. The 12·8% of bound magnesium found by Bialaszewicz is probably based on an error in the interpretation of the magnesium analysis. He determined magnesium in the filtrate from calcium estimations apparently as magnesium ammonium phosphate, and in the ashed plasma the copper from the haemocyanin would come down as phosphate and be included as 'magnesium'. This error would not arise in the protein-free ultrafiltrate. Robertson & Webb (1939) have taken copper into account in their hydroxyquinoline method for magnesium used in this paper.

IV. MOLLUSCA

Ionic regulation of blood. The figures in Table 4 reveal only slight regulation in the three lamellibranchs. Although all have considerably higher concentrations of potassium in their blood, *Ostrea* and *Mytilus edulis* are virtually in equilibrium with

Table 4. *Mollusca*

	Concentrations in plasma as percentage of concentration in dialysed plasma						mg./ml.	
	Na	K	Ca	Mg	Cl	SO_4	Protein	H_2O
Lamellibranchia								
Ostrea edulis	99·5	129·1	100·6	102·3	99·8	99·7	0·2	982
Mytilus edulis	100·0	134·7	99·5	99·5	100·5	98·2	0·3	984
M. galloprovincialis	100·9	120·8	106·6	96·5	98·6	120·0	0·8	984
Gastropoda								
Archidoris britannica	98·7	128·1	131·7	107·3	100·2	96·3	0·4	982
Cephalopoda								
Sepia officinalis	92·5	205·2	90·5	98·1	105·1	22·1	109	892

the external medium with respect to the other ions. The figures are from analyses of pooled plasma from ten and twelve specimens respectively. Accumulation of sulphate ions shown by the Mediterranean *M. galloprovincialis* is a rare feature in

marine invertebrates. In analyses of nearly forty invertebrates, this has been found only in the two species of *Eupagurus* and in *Hyas araneus*. The plasma from twenty specimens of the bivalve had a sulphate concentration 125% of that in the sea water of Naples aquarium. Ten individuals from the same batch were kept for 4 days in thoroughly aerated water, and the pooled plasma had a value of 115%. These high values were checked and confirmed by microgravimetric determinations of the sulphate as barium sulphate, in addition to the usual volumetric barium iodate procedure. While the meaning of the accumulation of sulphate ions is obscure, the consequences are slight alterations in sodium and chloride ions, the latter falling significantly owing to the presence of excess sulphate anions, and the sodium ions increasing slightly to maintain osmotic equilibrium in a solution containing more divalent sulphate ions than sea water.

Archidoris, a common opisthobranch, shows more ionic regulation than the bivalves, especially in its considerable accumulation of calcium (132%) and magnesium (107%). As in the other opisthobranch *Pleurobranchus* analysed previously (Robertson, 1949), the low protein content of *Archidoris* points to the absence of haemocyanin in this group, as already deduced by Webb (1937) from copper estimations. McCance & Masters (1937) have given figures for the 'mucus' secreted by *Archidoris* from which the visceral mass had been removed. Their figures appear to show considerably more ionic regulation in the formation of this fluid, but the 'mucus' would seem to have been a mixture of blood and mucus.

The peculiarities of ionic regulation in the plasma of *Sepia* are those already discovered in *Eledone* and *Loligo* (Robertson, 1949), a striking accumulation of potassium and a lowered sodium and sulphate, with a protein concentration exceeding 100 g./l. Separate analyses of three individuals, a male and two females, were made, the ranges of regulation being Na 92–94%, K 193–223%, Ca 84–97%, Mg 97–100%, Cl 105–106%, SO_4 17–29%. Of interest are the correlations between chloride, sulphate and sodium ions in the three cephalopods, a decreasing sulphate in the series *Eledone*, *Loligo* and *Sepia* being associated with increasing chloride and decreasing sodium. Granted a marked decrease in sulphate as a result of active regulation and maintenance of approximate osmotic equilibrium between the plasma and sea water, chloride ions must rise to balance the cations, while the cations themselves would decrease slightly. It can be calculated that in a synthetic sea water in which the sulphate is reduced to a fifth and the potassium doubled the chloride would be 104·7% and the sodium 93·8% of the values in an isosmotic sea water of normal composition. These values are approached in the analysis of *Sepia* plasma given in Table 4. With reduced sulphate but normal potassium, the sodium figure would be higher—95·9%.

It is probable that the excretory organs play a part in the ionic regulation of the plasma in cephalopods (Robertson, 1949). The fluid from the renal sac of the male *Sepia* had a protein concentration of only about 1 g./l. compared with 109 g./l. in the plasma. Moreover, it showed wide differences from the plasma or a plasma ultrafiltrate in all ions except chloride (Table 5). These differences are tending to lower the sulphate of the blood and raise the remaining potassium, calcium,

magnesium and chloride ions, but only in the cases of potassium, chloride and sulphate are these tendencies in line with the composition of the blood. The surprising finding that the sodium of the fluid was only 79% of the plasma sodium pointed to the presence of some other cation in order to balance the anions and make up the total concentration of ions to about that of the plasma. In a second *Sepia* the sodium of the renal sac fluid was 88% of the plasma concentration, in a third 92%; the latter had received an injection of sucrose for other purposes.

The missing cation proved to be ammonium which was measured by a micro-diffusion method (Conway, 1947). In the first specimen (Table 5) the amount was 2·64 mg. NH_4/g. water, 146 m.equiv./kg. water, which added to the sum of the other cations gave a concentration of 613 m.equiv. compared with 609 m.equiv. for chloride and sulphate. Electro-neutrality was also satisfied in the third *Sepia* which had 1·06 mg. NH_4/g. water, 58·7 m.equiv./kg. water, the total cations being 622 and anions 619 m.equiv. Figures for *Sepia* given by Delaunay (1931) are 1·12 and 1·19 mg. NH_4/ml. in the 'urine'.

Table 5. *Renal sac fluid of* Sepia officinalis

	mg./g. water							mg. ions/kg. water
	Na	K	NH_4	Ca	Mg	Cl	SO_4	
Plasma	10·58	0·931	—	0·434	1·383	20·87	0·47	1145
Renal sac fluid	8·35	0·465	2·64	0·302	0·936	20·85	1·01	1166
Sea water	11·31	0·409	—	0·432	1·364	20·38	2·845	1176*
Fluid as % plasma	79	50	—	70	68	100	215	102
Plasma ultrafiltrate† as % plasma	98	98	—	84	96	102	105	100

* Includes 2 mg. ions HCO_3.

† Calculated from dialysis experiments, using the mean Donnan ratio except in the case of calcium.

In *Eledone*, however, the sodium concentration in the renal sac fluid was on the average 2% higher than in the plasma, suggesting that the amount of ammonium in the fluid was negligible (Robertson, 1949). Delaunay (1931) gives two figures, 29 and 4 m.equiv./l. for *Octopus vulgaris*. The discrepancy between the *Sepia* and *Eledone* analyses may be connected with the different conditions of the animals when analysed. The *Sepia* were kept only 16–24 hr. after capture before analysis at Plymouth, and their high ammonium excretion may be considered to reflect recent feeding and breakdown of protein. *Eledone* specimens at Millport, on the other hand, were kept several days in the tanks without food, and their nitrogenous excretion had probably fallen to a low level.

An attempt was made to estimate the turnover in water and ions of a male *Sepia*. Fluid lost from the renal sacs must be balanced by uptake of water and ions from sea water, through the gills or via the gut. Isosmotic sucrose was injected into the anterior vena cava in front of the siphon, and 2 hr. later all the fluid in the renal sacs and samples of blood were collected. The sucrose in the two fluids was determined as the difference between total reducing substances before and after hydrolysis

with an invertase preparation, Somogyi's (1945) copper method being used for measurement of reducing power.

Weight of *Sepia*, 895 g.

Sucrose injected, 5 ml. of solution containing 332 g./kg. water, equivalent to 275 g./l. ($\Delta = 1{\cdot}99°$ C.).

Renal fluid collected, 7·0 ml. (containing 6·86 g. water).

Glucose-fructose equivalent of sucrose: plasma, 5·19 mg./g. water; renal fluid, 2·37 mg./g. water (= 45·6% plasma value).

Total glucose-fructose of 7 ml. renal fluid = $2{\cdot}37 \times 6{\cdot}86 = 16{\cdot}26$ mg.

Volume of plasma water containing this amount = $16{\cdot}26/5{\cdot}19 = 3{\cdot}133$ ml.

This is equivalent to a volume of 'urine' of $3{\cdot}133/0{\cdot}98 = 3{\cdot}20$ ml. (factor 0·98 since 1 ml. urine contains 0·98 ml. water).

Urine production = 3·20 ml./2 hr.
= 38·4 ml./24 hr. in a *Sepia* of 895 g.
= 42·9 ml./kg. 24 hr. (approx.).

The accuracy of this assessment of fluid turnover in *Sepia*, 4·3% of the body weight per day, depends on the correctness of a number of assumptions, namely, that sucrose is excreted by filtration only, that injection of 5 ml. fluid does not stimulate filtration, and that no sucrose-containing fluid escapes through the ureters before collection at the end of a 2 hr. period. The latter assumption especially is rather hazardous, and the figure of 4·3% may perhaps be regarded as a minimal one.

From this experiment an estimate can also be made of the total volume of extracellular fluid, taking sucrose as a substance which will not diffuse significantly into cells. The calculation is made by dividing the glucose-fructose equivalent of the 1·375 g. sucrose injected by the plasma equivalent.

Volume of extracellular fluid $= \frac{1{\cdot}375 \times 1000 \times 1{\cdot}05 \text{ ml.}}{5{\cdot}19} = 278$ ml. (the factor of 1·05 depends on the fact that 1 g. sucrose gives 1·05 g. glucose-fructose on hydrolysis).

Percentage extracellular fluid $= 278/895 \times 100 = 31{\cdot}1\%$. Correction for the facts that the 14 ml. vitreous humour from the eyes and the 7 ml. fluid from the renal sacs had respectively concentrations 2 and 46% of the glucose-fructose level of the plasma increases this percentage to 33·0%. Unfortunately, it has been found that sucrose appears to enter muscle cells to some extent in the crustacean *Nephrops*, and this may well be the case in *Sepia*, making this volume of 33·0% an overestimate. In mammals sucrose is considered one of the best indicators of extracellular space (Wilde, 1945; Kruhøffer, 1946), but it appears to penetrate slowly amphibian muscle cells (Krogh & Lindberg, 1944).

Excretion of a fluid amounting to 4% of the body weight per day implies a turnover of water equivalent to the volume of the extracellular fluid every 8 days. From what has been said about possible errors, 8 days is probably an overestimate.

Eye-fluids of cephalopods. The many structural similarities between the cephalopod and the vertebrate eye have long been regarded as an outstanding example of

convergence in evolution. Certain non-sensory aspects of the cephalopod eye are investigated here. In both types of eye a fluid is present in front of the lens, the aqueous humour; behind it and in front of the retina in the vertebrate eye is the vitreous body, a gel, and in the cephalopod the liquid vitreous humour. Ophthalmic arteries supply the retina, ciliary body and iris. In mammals considerable attention has been given to the mechanism of formation of the aqueous humour, particularly the problem of how far it is an ultrafiltrate or dialysate of the plasma in the capillaries of the ciliary body. Varying conclusions have been reached, depending often on which ions or non-electrolytes have been considered. Apart from the chloride analyses of Derrien (1938), who found the concentrations of this ion in plasma and vitreous fluid to be the same, no data on the composition of the eye-fluids seem to exist for cephalopods. Analyses were therefore made of the plasma, vitreous and aqueous humours, the sea water with which the animals were in equilibrium, and samples of plasma dialysed against sea water.

Table 6. *Eye-fluids of cephalopods*

	mg. ions/kg. water							m.equiv.	
	Na	K	Ca	Mg	Cl	SO_4	Total	Cations	Anions
Sepia									
Plasma	465	21·9	11·6	57·7	591	6·3	1153	626	604
	±3	±1·2	±0·6	±0·6	±2	±1·2	±6		
Vitreous humour	533	13·5	13·1	5·4	575	3·1	1144	584	581
	±2	±0·5	±0·4	±1·2	±4	±0·0	±6		
Sea water	492	10·5	10·8	56·1	575	29·6	1174*	636	634*
Sepia†									
Aqueous humour	561	13·5	12·0	53·8	672	13·8	1326	706	700
Vitreous humour	627	16·3	14·2	7·0	665	6·4	1336	686	678
Sea water	564	12·0	12·4	64·3	659	34·0	1344*	729	726*
Loligo									
Plasma	419	20·6	11·3	51·6	522	7·3	1032	565	537
	±5	±2·2	±0·2	±1·1	±5	±0·9	±11		
Vitreous humour	480	22·6	4·1	8·7	519	3·4	1037	528	526
	±5	±4·1	±0·2	±0·8	±2	±0·3	±2		
Sea water	432	9·2	9·5	49·4	506	26·0	1032*	560	558*
Eledone									
Plasma	438	13·0	11·0	54·6	513	20·7	1050	582	554
Vitreous humour	491	15·9	15·2	10·5	485	36·6	1054	558	558
Sea water	436	9·3	9·6	49·8	510	26·3	1041*	565	563*

* *minus* HCO_3 of 2–3 mg. ions or m.equiv.
† Naples specimen.

Seven plasma-vitreous fluid comparisons are summarized in Table 6 in which are given the mean figures with mean deviations for three *Sepia officinalis* and three *Loligo forbesi*, together with a single analysis of *Eledone cirrosa*. A striking feature is the wide difference between the two fluids in practically every ion. Magnesium shows a remarkable decrease in the vitreous humour in the three genera, falling even to 10% of the level in the plasma in *Sepia*. Compensating increase of sodium ions seems to adjust the cation-anion balance and maintain the total ionic concentration which is within 1% of that of the plasma. Chloride falls definitely in *Sepia* and *Eledone*. Most of the other ions in the vitreous humour are lower than in the

plasma, except calcium in *Sepia*, potassium in *Loligo*, and potassium, calcium and sulphate in *Eledone*. In both *Sepia* and *Loligo* the mean deviations from the average figures are small.

Cation-anion balance, as determined by summing the principal ions, is satisfactory (Table 6). The deficiency of anions in the plasma, 22–28 m.equiv., is probably made up chiefly by protein, the concentration of which in g./kg. water was 122 ± 4 in *Sepia*, 173 ± 6 in *Loligo*, and 117 in *Eledone*. Heat-coagulable protein is practically absent from the clear vitreous humour, a value of only 0·24 g./kg. water being obtained in *Sepia*. The contribution of bicarbonate and phosphate to the anions of marine invertebrate body fluids is small. Several estimations of these were made, bicarbonate by Conway's (1947) method, and inorganic phosphate according to Sumner (1944). In *Eledone* plasma bicarbonate was only 4 m.equiv., and the pH as determined by the glass-electrode 7·10 (15° C.). This was blood collected under oil from the dorsal aorta; the pH of vitreous humour collected from

Table 7. *Eye-fluids of cephalopods*

	Concentrations as percentage of blood values					
	Na	K	Ca	Mg	Cl	SO_4
Sepia						
Vitreous humour	115	62	113	9	97	49
Dialysate of plasma*	98	98	84	96	102	105
Loligo						
Vitreous humour	115	110	36	17	99	47
Dialysate of plasma†	98	98	86	96	102	104
Eledone						
Vitreous humour	112	122	138	19	95	177
Dialysate of plasma*	99	99	92	98	101	102

* The mean Donnan ratio has been given, except in the case of calcium.
† Only ultrafilterable calcium analysed; for other ions a Donnan ratio of 1·02 assumed.

the same specimen was 6·70. Inorganic phosphorus comparisons in plasma and vitreous fluid were *Loligo*—0·094 and 0·057 mg. P/ml., *Sepia*—0·053 and 0·035 mg. P/ml. Such figures represent 1–3 mg. ions and 2–5 m.equiv. $H_2PO_4^- + HPO_4^{2-}$.

Taking into consideration the data in Tables 6 and 7, it is quite evident that the vitreous fluid is far from being a dialysate of the plasma in these three cephalopods. Apart from the fact that it is virtually a protein-free fluid of approximately similar osmotic pressure, it differs widely from a dialysate in respect of each of the principal ions and of inorganic phosphate. The equilibrium here between the plasma and vitreous fluid would seem to be a steady state in which all the ions are actively maintained, presumably by processes of secretion and absorption by the cells of the retina.

The aqueous humour in front of the lens exists in small quantities, and it has been examined only in *Sepia*. In this animal a small pore in the cornea allows communication between this fluid and the external environment (Tompsett, 1939). That the aqueous fluid is not sea water is at once apparent from the analyses of the eye-fluids from a Naples specimen shown in Table 6. Neither is it a dialysate of the

plasma, but in most respects it is intermediate in composition between the vitreous fluid and sea water. Its composition cannot be explained simply as a mixture in certain proportions of a plasma ultrafiltrate and sea water; magnesium in such a case would be at a higher level. Diffusion between the aqueous and vitreous fluids is a possibility, but judging from the respective compositions of the two fluids must be very restricted. Two hypotheses may be suggested:

(1) The aqueous fluid is a plasma filtrate modified by the secretion of extra Na^+, Ca^{2+} and SO_4^{2-} ions, and reabsorption of K^+, Mg^{2+} and Cl^- ions by the epithelia lining the anterior chamber.

(2) The aqueous fluid is chiefly sea water from which some Mg^{2+} and SO_4^{2-} ions have been absorbed by the epithelia, and into which have diffused from the blood small quantities of other ions in accordance with the concentration gradient.

It might perhaps be argued against (1) that it is unlikely that cellular activity of the epithelia and retina in adjacent parts of one organ would produce fluids of such different composition as the aqueous and vitreous humours. The second hypothesis implies a steady state: sea water entering through the corneal pore would have to be removed continuously to prevent the concentrations of ions other than Mg^{2+} and SO_4^{2-} from reaching levels characteristic of a dialysate of the plasma. Further work is necessary before the origin of the aqueous humour can be definitely established.

Certain deductions can be made about the forces concerned in maintaining the water balance of the vitreous humour. The colloid osmotic pressure due to the haemocyanin of the blood, and hydrostatic pressure may be important if the blood and vitreous humour have osmotic pressures similar to those of a Donnan equilibrium, although such an equilibrium in respect of each ion is not found. To prevent fluid being withdrawn by the plasma through the retina, the hydrostatic pressure in the retinal capillaries must either equal or exceed the colloid osmotic pressure. If the retinal cells are able, by transporting ions, to raise the concentration of osmotically active particles in the vitreous fluid slightly above that of the plasma, water would tend to pass across the retina from the capillaries, keeping up the turgor in the interior of the eye.

The data in Table 6 are not sufficiently exact to decide whether the vitreous humour is slightly hyperosmotic to the plasma. Such slight differences as exist are within the error of the summation method of determining total ionic concentration, which is about 1% ($3 \times$ S.D.).

V. DISCUSSION

The further data on ionic regulation given in this paper confirm and amplify the previous findings of Robertson (1949). Animals of the echinoderm and lamellibranch groups, *Holothuria* and the bivalves *Mytilus* and *Ostrea*, show little ionic regulation in their coelomic fluid or plasma, apart from the accumulation of potassium. Magnesium remains within 3 or 4% of the equilibrium value, while calcium sometimes exceeds this by a few per cent. *Phascolosoma* differs from the polychaetes in having the magnesium concentration of the coelomic fluid reduced to

about 70%, and a corresponding increase of the sodium concentration to 104% is necessary to maintain the balance of cations and osmotic equilibrium. Calcium and magnesium are accumulated to a considerable extent in *Archidoris*, a feature distinguishing it from the lamellibranchs, but protein is still very low owing to the absence of haemocyanin in this gastropod.

High concentrations of protein and regulation of every ion characterizes *Sepia* and all the Crustacea examined. With respect to Mg^{2+} and SO_4^{2-} ions, the eight crustaceans isosmotic with sea water show all possible combinations: high Mg^{2+} and SO_4^{2-}, e.g. *Hyas*; high Mg^{2+} and low SO_4^{2-}—*Dromia*; reduced Mg^{2+} and SO_4^{2-}, e.g. *Squilla*; accumulation of SO_4^{2-} with low Mg^{2+}—*Eupagurus*. Whatever were the combinations, the concentration of Na^+ ions was apparently altered in such a way as to maintain electroneutrality and total ionic concentration. The usual finding was a rise in Na^+ ions to compensate for loss of Mg^{2+}. Where SO_4^{2-} ions were reduced and Mg^{2+} maintained (*Dromia*), Cl^- ions increased but Na^+ ions fell to 97% of the dialysis value. This finding, at first surprising, is nevertheless in harmony with the maintenance of osmotic equilibrium, since Na^+ ions paired with Cl^- ions are more 'active' than when paired with SO_4^{2-} ions.

The excretion of ammonium ions by the renal organs of *Sepia* in amounts sometimes as great as 146 m.equiv./kg. water or 24% of the total cation-equivalents presents another problem in osmotic regulation. It is met by a reduction in the other cations excreted, so that the total concentration of the ions in the fluid from the renal sacs is approximately equal to that of the plasma. In the specimen with 146 m.equiv. or mg. ions NH_4, these concentrations were within 1·8% of each other. When NH_4^+ ions are excreted in quantity adjustment of osmotic pressure by diminished excretion of other cations falls mainly on the Na^+ ion, since in mg. ions it constitutes about 84% of the total plasma cations.

The rate at which fluid is excreted by the renal organs in cephalopods would illustrate the importance of this excretion in regard to ionic regulation. In the literature the only data seem to be those of von Fürth (1900), who found the daily urine of a large *Octopus* of 4 kg. to be 80 ml., representing an excretion of 2% of the body weight per day. His method consisted in ligaturing the ureters, and this figure may be a minimal value as filtration might be expected to be reduced if pressure were to build up in the renal sacs. The figure of 4·3% obtained in this paper for *Sepia* is somewhat uncertain, but may be of the right order of magnitude. This would amount to the excretion of a volume of fluid equal to that of the extracellular fluid (33% of the body weight) within 8 days. A comparable calculation for the crab *Carcinus maenas* with 35% extracellular fluid and an output of 5% of the body weight per day is 7 days (Webb, 1940).

The peculiarities in ionic composition of the vitreous humour of the eyes of *Eledone*, *Loligo* and *Sepia* demand the expenditure of energy in effecting and sustaining the marked differences in this fluid from a dialysate of the plasma. Every ion is maintained at a level different from what it would be if there were physicochemical equilibrium with the plasma across the retina. Magnesium concentrations in the vitreous fluid of 10–20% of those of the plasma on the other side of the

retina are especially noteworthy, but what bearing this has on the sensory perception of this layer is unknown. A relative impermeability of the blood-vitreous fluid barrier is necessary for the maintenance of such differences of concentration, and this was found to be the case for sucrose injected into the plasma. Two hours later the concentration of sucrose in the vitreous humour was only 2% of that in the plasma.

Detailed comparison with the mammalian eye is not possible owing to the inadequacy of the chemical data for any one mammalian species (e.g. Davson, 1949; Duke-Elder & Goldsmith, 1951). It would seem, however, that concentrations of ions such as sodium, potassium and chloride in the aqueous humour and vitreous gel are only slightly different from those of a true dialysate of the plasma. There are indications that magnesium of the vitreous body may be considerably lower than the plasma magnesium, at least in the horse (Duke-Elder, 1929). The vitreous fluid

Table 8. *Relative ionic composition of body fluids*

	Weight units (g.)						Equivalents		
	Na	K	Ca	Mg	Cl	SO_4	$\frac{Na+K}{Ca+Mg}$	$\frac{K}{Mg}$	$\frac{Ca}{Mg}$
Sea water	55·5	2·01	2·12	6·69	100	14·0	3·8	0·09	0·19
Holothuria	56	2·06	2·15	6·9	100	13·9	3·7	0·09	0·19
Phascolosoma	58	2·24	2·25	4·7	100	12·9	5·2	0·15	0·29
Ostrea	55	2·59	2·14	6·9	100	13·9	3·7	0·12	0·19
Mytilus edulis	55	2·70	2·10	6·6	100	13·6	3·8	0·13	0·19
M. galloprovincialis	57	2·46	2·29	6·5	100	17·0	3·9	0·12	0·21
Archidoris	55	2·57	2·79	7·2	100	13·4	3·3	0·11	0·24
Sepia	51	4·10	2·22	6·7	100	2·9	3·5	0·19	0·20
Eupagurus bernhardus	64	2·88	3·75	3·7	100	19·0	5·8	0·24	0·61
E. prideauxi	61	3·38	3·38	5·3	100	17·7	4·5	0·20	0·39
Dromia	53	2·40	2·04	6·7	100	7·0	3·7	0·11	0·19
Hyas	57	2·68	2·52	6·4	100	14·0	3·9	0·13	0·24
Maia	57	2·58	2·90	5·7	100	8·8	4·2	0·14	0·31
Portunus depurator	61	2·82	2·67	3·3	100	11·1	6·7	0·26	0·49
P. puber	63	3·02	2·92	2·8	100	11·3	7·4	0·33	0·63
Pachygrapsus	62	2·24	2·55	1·9	100	7·3	9·7	0·36	0·81
Squilla	64	2·69	2·59	2·2	100	11·2	9·1	0·37	0·70

of cephalopod eyes is far from being a true dialysate of the plasma, and the marked differences are presumably due to secretion and absorption of ions by the retina. In *Sepia* the aqueous humour has a composition quite unlike the vitreous humour or a plasma dialysate but more like sea water; its precise origin has not been established.

In Table 8 is given the relative ionic composition of the plasma or coelomic fluid of the animals which have been analysed, based on a chloride value of 100; three ionic ratios of equivalents have also been calculated. Several points may be emphasized: the general resemblance to sea water of the coelomic fluid of *Holothuria* and the plasma of the bivalves; the high potassium and low sulphate of *Sepia*; the increase of sodium where magnesium is reduced in *Phascolosoma* and most of the crustaceans, with corresponding changes in the ionic ratios; the high sulphate values of *Mytilus galloprovincialis* and the two species of *Eupagurus*.

In a series of six crustaceans, it was found previously (Robertson, 1949) that the more active ones had low concentrations of magnesium in the blood. Further data have enabled Fig. 1 to be drawn, illustrating the values of magnesium in sea water and the plasma of sixteen crustaceans. Of these sixteen any one familiar with them would pick out without hesitation the two spider-crabs *Maia* and *Hyas*, the anomuran *Lithodes*, and *Dromia* as being the least active and those which respond most slowly to mechanical stimulation. It is just these four genera that have the highest magnesium values, 84–101 % of that in sea water. The two hermit-crabs are anomurans like *Lithodes* but more active, and they have correspondingly lower

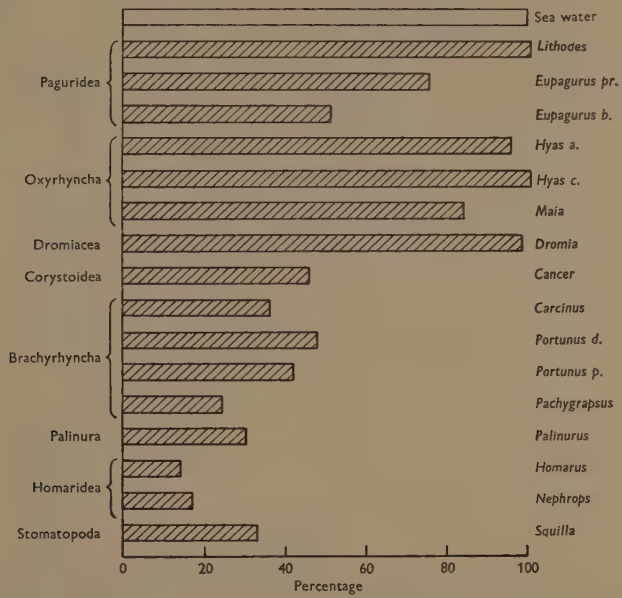

Fig. 1. Comparison between magnesium values in crustacean plasma and sea water (on mg./g. water basis). *a. araneus, b. bernhardus, c. coarctatos, d. depurator, pr. prideauxi, p. puber.*

concentrations of magnesium. The remaining crabs (with one apparent exception), the lobsters and *Squilla* are much more active, and all have magnesium values less than half that in sea water. *Cancer* might be considered anomalous as in aquaria it usually shows little activity; but when displaced from a preferred corner, it can run quite rapidly back.

When it is recalled that excess of magnesium ions in the form of solutions of magnesium chloride or sulphate are used to narcotize marine animals (see e.g. Pantin, 1946), there seem grounds for assuming that reduction in magnesium and increased activity are causally related. From this standpoint *Dromia*, *Lithodes* and the spider-crabs might be considered as living in a semi-narcotized state. Evidence

from the study of the isolated walking legs of *Carcinus* indicates that perfusion with a fluid containing 1·5–2 times the blood concentration of magnesium depresses neuromuscular transmission (Katz, 1936), and perfusing with a fluid containing only 5–20% of the blood concentration enhances the submaximal muscular response (Boardman & Collier, 1946). Findings that the mechanical response to nerve stimulation varies inversely with the magnesium concentration in the perfusing fluid have also been reported in three other decapods, *Maia*, *Panulirus* and *Cambarus* (Waterman, 1941).

However, other ions as well as magnesium influence activity, and the balance between certain ions is often important. Bethe (1929) found that the magnesium concentration of the blood of *Carcinus* increased to a value over three times the normal when the external magnesium was increased by about the same factor, resulting in the lessening of muscular tone and impairment of the normal reflexes. Exactly similar results were found when the calcium concentration of the blood was reduced; but crabs became very excitable when the plasma value of calcium was increased by keeping them in calcium-enriched sea water. Thus the balance between calcium and magnesium is probably important; the ratio of the equivalents of Ca/Mg ranges from 0·19–0·31 in the *Lithodes-Dromia*-spider-crab group to 0·39–2·0 in the remaining decapods and *Squilla* (Table 8; Table 9, Robertson, 1949). It may be recalled that anaesthesia produced by injection of Mg^{2+} ions in mammals is counteracted by injection of Ca^{2+} ions (Heilbrunn, 1947, p. 460).

Wide variations in organization and activity exist among members of the Mollusca, a phylum in which magnesium is maintained at a uniformly high level. Calcium in the blood does not vary very much, although it is usually slightly higher than the value in sea water. The most variable cation is potassium. Moderate increases in potassium ions have a stimulatory action on the neuromuscular system (e.g. Wells, 1928; Ross & Pantin, 1940). Bethe (1927) found that increase in potassium in the external medium had similar effects to increase in calcium on medusae, *Phoronis* worms and various crustaceans, these ions acting synergically in augmenting rhythmical movements. It is therefore suggested that the pre-eminence in muscular activity of members of the Cephalopoda compared with the Lamellibranchia or Gastropoda, although based primarily on structural characters, may be at least enhanced by the very high potassium concentrations maintained in the blood by members of this class.

Undoubtedly factors other than ionic composition of the blood may influence activity, such as hormones. Florkin (1949) relates the torpor of *Cancer* and the nimbleness of *Carcinus* to differences in the blood-sugar content, high in the former and low in the latter (Roche & Dumazert, 1935; Florkin, 1937), but no supporting evidence is given for assuming a direct effect of the level of blood sugar on activity. Indeed, other authors (Gray & Hall, 1930) have found an opposite correlation in marine fishes, the most active having high blood sugars.

VI. CONCLUSIONS

Certain general conclusions can be drawn from the present data and those of Robertson (1949), which consist of analyses of the mesogloea of a coelenterate and of the plasma or coelomic fluid of thirty-four marine invertebrates belonging to the Echinodermata, Annelida, Sipunculoidea, Mollusca and Arthropoda. All these animals, with the exception of the grapsoid crab *Pachygrapsus*, are in osmotic equilibrium with sea water, within 1–2%. To a varying degree ionic regulation exists in all the animals examined, ranging from a slight accumulation of potassium in the Echinodermata to regulation of every ion in the Cephalopoda and most of the Crustacea.

Ionic regulation is slight in the more simply organized, relatively inactive coelenterates, echinoderms, polychaetes, lamellibranchs and gastropods. All these, excepting the prosobranch gastropods, have very low protein contents in their body fluids (or mesogloea in *Aurelia*), usually below 1 g./l. Pronounced regulation is shown by the more active, highly organized Crustacea and Cephalopoda, all of which contain the respiratory pigment haemocyanin; high concentrations of protein are characteristic, 29–80 g./l. in the decapods and 105–150 g./l. in the cephalopods.

Where there is ionic regulation of the blood, the equilibrium which exists across gills and other permeable membranes is a steady state in which the concentrations of ions are maintained actively. Replacement of these membranes by one of collodion results in a physico-chemical equilibrium in which Donnan forces come into play. The Donnan ratio for sodium, potassium, magnesium, chloride and sulphate does not exceed 1·03 in the decapod Crustacea and the cephalopod Mollusca. In these animals none of the cations is bound to protein except calcium, of which 10–20% exists as a calcium-protein complex.

Active regulation exists also in the fluids produced by the excretory organs of decapods and cephalopods, and in the eye-fluids of the latter.

The mechanism of ionic regulation of the plasma in decapods and cephalopods involves the continuous selective excretion of ions in the excretory fluids and controlled uptake of ions by the permeable surfaces.

The concentration of magnesium in the blood of decapod crustaceans is related to activity; those animals with high levels are slow-moving and inactive, whereas those with low levels are capable of quick movement and are generally more active. Cephalopods have high levels of potassium ions which may contribute to their powers of active movement.

Variations of ionic regulation in different specimens of a species are small in the few cases investigated. In general, potassium is the most variable ion; sodium and chloride are the least variable, as is to be expected, since they form such a large proportion of the total concentration of ions which is maintained at the same level as the external medium in all the animals except one.

VII. SUMMARY

1. The blood or coelomic fluid has been analysed in sixteen marine invertebrates to determine the amount of ionic regulation.

2. Little regulation is shown by *Holothuria* and the bivalves *Ostrea* and *Mytilus* except in potassium. The bivalves accumulate potassium (up to 135% of the concentration in sea water), and *Mytilus galloprovincialis* accumulates sulphate (to 120%), an unusual feature.

3. The nudibranch *Archidoris* accumulates potassium (128%), calcium (132%) and magnesium (107%), while the sipunculid *Phascolosoma* has lower magnesium (69%) and sulphate (91%) but higher sodium (104%).

4. The cephalopod *Sepia* regulates all its ions except magnesium, range of values (expressed as percentage of concentration in dialysed plasma) being Na 92–94%, K 193–223%, Ca 84–97%, Mg 97–100%, Cl 105–106%, SO_4 17–29%. Protein averages 109 g./l. in the three specimens analysed. Fluid from the renal sacs contains high concentrations of NH_4^+ ions, in two specimens 146 and 59 m.equiv./kg. water, and differs from a plasma ultrafiltrate in the concentration of all other ions.

5. The vitreous humour in the cephalopod eye is a clear protein-free fluid, isosmotic with the plasma within 1% but having ionic concentrations markedly different from those of a plasma ultrafiltrate or dialysate. In *Sepia*, *Loligo* and *Eledone* magnesium may be only 10–20% and sodium over 115% of the concentrations in a dialysate of the plasma. In one specimen of *Sepia* the aqueous fluid in front of the lens largely resembled sea water except for lower concentrations of magnesium and sulphate.

6. Among the decapod and stomatopod Crustacea regulation of all ions exists, ranges in eight species being Na 97–111%, K 120–156%, Ca 84–137%, Mg 32–99%, Cl 96–103%, SO_4 53–135%. Species of *Portunus* and *Eupagurus* show more regulation than *Dromia* and the spider-crabs *Maia* and *Hyas*. Regulation in the stomatopod *Squilla* resembles that in the portunid family. In the grapsoid crab *Pachygrapsus* each ion in the plasma is maintained below its equilibrium value: Na 94%, K 95%, Ca 92%, Mg 24%, Cl 87%, SO_4 46%; total ions 1·163 g. ions/kg. water compared with 1·353 in sea water.

7. In sixteen crustaceans an inverse relationship exists between the degree of activity and the magnesium content of the blood: the more active ones have low values of magnesium.

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